Putative tumour suppressor gene necdin is hypermethylated and mutated in human cancer

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Background: Necdin (NDN) expression is downregulated in telomerase-immortalised normal human urothelial cells. Telomerase-immortalised normal human urothelial cells have no detected genetic alterations. Accordingly, many of the genes whose expression is altered following immortalisation are those for which epigenetic silencing is reported.

Methods: NDN expression was examined in normal tissues and tumour cell lines by quantitative real-time PCR and immunoblotting. Immunohistochemistry was performed on urothelial carcinoma (UC). Urothelial carcinoma and UC cell lines were subject to HumanMethylation27 BeadChip Array-based methylation analyses. Mutation screening was performed. The functional significance of NDN expression was investigated using retroviral-mediated downregulation or overexpression.

Results: NDN protein was widely expressed in normal tissues. Loss of expression was observed in 38 out of 44 (86%) of UC cell lines and 19 out of 25 (76%) of non-UC cell lines. Loss of NDN protein was found in the majority of primary UC. Oncomine analysis demonstrated downregulation of expression in multiple tumour types. In UC, tumour-specific hypermethylation of NDN and key CpG sites where hypermethylation correlated with reduced expression were identified. Six novel mutations, including some of predicted functional significance, were identified in colorectal and ovarian cancer cell lines. Functional studies showed that NDN could suppress colony formation at low cell density and affect anchorage-independent growth and anoikis in vitro.

Conclusion: NDN is a novel tumour suppressor candidate that is downregulated and hypermethylated or mutated in cancer.
derived from the cells lining the bladder, the remainder of cases are squamous cell carcinoma or adenocarcinoma. Rarely, sarcoma or small-cell carcinoma are observed.

Integration of expression and hypermethylation data has allowed identification of key CpG sites involved in transcriptional silencing. Oncomine expression microarray data were examined to determine the expression of NDN transcript in multiple tumour types compared with normal tissue. The functional significance of NDN expression was examined in vitro using retroviral-mediated transduction. We present the first evidence to support our hypothesis that NDN is a potential tumour suppressor gene with a role in multiple tumour types (Chapman and Knowles, 2009).

**MATERIALS AND METHODS**

**Immunohistochemistry (IHC).** Necdin IHC was optimised using anti-mouse NDN (AB9372, Millipore, Watford, UK). A total of 1 × 10⁶ NHUCs (NDN positive), TERT-NHUC-pFBhy (NDN low/ negative) and TERT-NHUC pFBhy-NDN (ectopic overexpression) were pelleted and 5 ml of 4% formalin/PBS were added overnight. Formalin was replaced with 70% ethanol. Pellets were embedded into 3% agarose, formalin fixed and paraffin embedded. Additional controls were brain, bladder, ureter or UC with known NDN transcript level. Deparaffinised and rehydrated sections were treated with 3% hydrogen peroxide. Antigen retrieval was by pressure cooking with citric acid buffer pH 6 for 2 min, Avidin Biotin blocking kit, catalysed signal amplification system (Dako, Ely, UK), followed by counterstaining with haematoxylin and eosin and cosin was performed.

Immunohistochemistry was performed on normal tissue microarray (TMA) II (Provitro, Berlin, Germany) or sections from Leeds Multidisciplinary Research Tissue Bank. Necdin expression was classified as strong (3), moderate (2) weak (1) or absent (0). Immunohistochemistry was performed using Menapath X-cell Plus polymer HRP detection kit (Menarini diagnostics, Wokingham, UK). A preliminary panel contained 72 paraffin-embedded UC from patients at St James’s University Hospital, U.K. Tumour samples were obtained with informed consent and the approval of the Local Research Ethics Committee and graded and staged according to the 1973 World Health Organization recommendations and T.N.M classification.

Tissue microarrays contained triplicate cores from 94 tumours from patients at Hospital Guadalajara and Hospital Central de Asturias, Spain. Three observers scored and discrepancies were resolved to give a single score for each core or section. For TMAs, where triplicate cores from a tumour had differing scores, if staining for a potential tumour suppressor gene, the lowest scoring core was recorded. Conversely, the highest scoring core was recorded for potential oncogenes. This principal was also applied if whole sections showed non-homogenous staining.

**Cell culture.** In all, 40 of 45 bladder cell lines are previously described (Yeager et al, 1998; Sarkar et al, 2000), LUCC6, LUCC7 and LUCC8 were derived from LIMM from G3pTa, G3pT2/3 and G2pTa UC, respectively. HCV29 was derived from non-malignant transitional epithelium from a patient who had received radiation therapy for UC (Bean et al, 1974) and SVHUC are in vitro transformed urothelial cells (Christian et al, 1987). Culture media used were as follows: Keratinocyte growth medium kit 2 (C-20111, Promocell, Heidelberg, Germany) with 0.09 mm CaCl₂, 3 mm glycerine and 1% FCS (LUCC6 and LUCC8). LUCC7 was grown as described (Sarkar et al, 2000) plus 30 ng ml⁻¹ cholera toxin (Sigma-Aldrich, Dorset, UK). NHUC, TERT-NHUC and LUCC cell lines (excluding LUCC2) were cultured on Primaria surfaces (BD Biosciences, Oxford, UK). Twenty-five non-UC cell lines and culture media are described in Supplementary Table 1. MMG1 primary acral melanoma cell line was from Professor Akifumi Yamamoto (Saitama Medical University International Medical Centre). SMYM-PRGP melanoma cells were from Dr Hiroshi Murata (Shinshu University School of Medicine, Japan; Murata et al., 2007).

Cell line identity was verified by short-tandem repeat DNA typing using Powerplex 16 kit (Promega, Southampton, UK). Profiles were compared with publically available data (ATCC, DSMZ). Where no reference profile was available it was confirmed that the profile did not match any cell line in the DSMZ database. All cell lines are routinely tested with PCR Mycoplasma Test Kit III (PromoKine, Heidelberg, Germany). NHUC and TERT-NHUC were derived as described (Chapman et al, 2006).

**Quantitative real-time PCR (QRT-PCR).** RNA was extracted from cell lines using RNeasy mini kit (Qiagen, Crawley, UK) or from frozen sections using Picopure RNA isolation kit (Arcturus Bioscience, Mountain View, CA, USA). cDNA was synthesised and expression of NDN was quantified using TaqMan QRT-PCR (assay Hs00267349_s1) and SDHA (Hs00417200_m1) (Applied Biosystems, Warrington, UK). Reference samples were pooled NHUC cDNA (for UC) or normal human adult universal cDNA (Source Bioscience LifeSciences, Nottingham, UK) for non-UC cell lines.

**Western blot.** A measure of 15 μg total protein was separated in 12% polyacrylamide gels. Antibodies used were anti-Necdin (07–565, Millipore) and anti-α-tubulin (MCA77G, AbD Serotec, Oxford, UK). DSH1 and RT4 did not express α-tubulin; therefore, blots were re-probed with anti-actin (Santa Cruz, Santa Cruz, CA, USA; data not shown).

**Methylation analysis.** Sequenom massARRAY EpiTYPER assay was used to screen NDN for CpG hypermethylation in TERT-NHUC and isogenic NHUC. Amplicons were designed using the EpiDesigner programme (http://www.EpiDesigner.com; Supplementary Table 2). Assay was performed at Sequenom (Hamburg, Germany) using standard protocols described in the MassARRAY EpiTYPER Application Guide (http://www.sequenom.com).

Methylation detection in UC cell lines and primary tumours was performed using Illumina HumanMethylation27 BeadChip arrays by Cambridge Genomic Services (University of Cambridge, Cambridge, UK) according to Illumina’s Infinium II Methylation protocol. DNA was extracted from 42 fresh frozen TaG2 UC with minimal genomic alteration (<2% genome altered by aCGH; Platt et al, manuscript in preparation) and 45 bladder cell lines using QIAamp DNA kit (Qiagen). Five hundred nanograms of DNA was bisulphite modified using EZ DNA methylation kit (Zymo, Irvine, CA, USA) and applied to the chip. Gene annotation was performed using the Mar 2006 (NCBI/26/hg18) assembly at the UCSC database (http://genome.ucsc.edu/). The array returns a β-value for each probe, which is representative of the average level of methylation, where 0 is no methylation and 1 is 100% methylation. Necdin is a maternally imprinted gene, therefore, control samples were from patients at Hospital Guadalajara and Hospital Central de Asturias, Spain. Three observers scored and discrepancies were resolved to give a single score for each core or section. For TMAs, where triplicate cores from a tumour had differing scores, if staining for a potential tumour suppressor gene, the lowest scoring core was recorded. Conversely, the highest scoring core was recorded for potential oncogenes. This principal was also applied if whole sections showed non-homogenous staining.

**Gene expression array profiling.** Whole-genome expression profiling (Hurst et al., manuscript in preparation) was performed using GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix, High Wycombe, UK). A measure of 5 μg of total RNA was labelled using the WT-Ovation Pico Target Prep v1.0 system (NuGen Technologies, Leek, The Netherlands) and hybridised according to the array manufacturer’s instructions. Arrays were scanned and CEL files imported into Partek Genomics Suite 6.5. Data were normalised and probe intensity measures were
generated using the Robust Microarray Analysis algorithm (Irizarry et al., 2003).

**Sequencing of NDN.** Ten microlitre of PCR reactions contained: 10 ng DNA, 4 pmol of each primer, HotStarTaq mastermix (Qiagen) and 10% DMSO. For melanoma cell lines, 10 ng BSA was added. Cycling conditions were: 15 min at 95°C then 36 cycles of 30 s at 95°C, 30 s at 57°C, 1 min at 72°C and a final cycle at 72°C for 10 min. The single exon of NDN was amplified in two fragments using primers: 1 F 5'-GAAGAGCTCCTGGACGCA GA-3' and 1 R 5'-TCAGCGCCACCTCCTGACG-3', 2 F 5'-TCA TCCTCGCCGGGGTGTTACG-3' and 2 R 5'-GTGAGGGTCAAGA AACCATTC-3'. Unincorporated primers and deoxynucleotides were removed using shrimp alkaline phosphatase and exonuclease I. Sequencing reactions were carried out using the corresponding primers and a BiDye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were compared with NCBI reference sequence NG_009380.1. Mutation nomenclature uses A of ATG as nucleotide 1.

**Retroviral transduction.** MGC/IMAGE Clone 1697/3347128 (Geneservice, Cambridge, UK) was sub-cloned as an EcoR1-Xho1 fragment into pFB Hydro (adapted from pFB neo from Agilent, Cheshire, UK). Oligos targeting NDN (Supplementary Table 3) or a non-silencing control oligo (Douglas et al., 2008) were cloned into pRetroSuper-puro (Tomlinson et al., 2007). Constructs were transfected into PhoenixIA (ATCC) using Mirus TransIT-293 (Cambridge Bioscience, Cambridge, UK). After 48 h, medium was harvested and filtered through a 0.4-μm filter. Supernatant containing 8 μg ml⁻¹ of polybrene (Sigma-Aldrich) was added to sub-confluent target cells for 6 h. After 48 h, cells were transferred into selection medium.

**Phenotypic assays.** For growth curves, cells were plated at 3.1 × 10⁴ cells per cm². To assess anchorage-independent growth, 4.2 × 10³ cells per cm² were plated in 0.3% Noble agar (Sigma-Aldrich) in medium onto a base of 0.6% agar. Cells were fed weekly with 0.3% agar/medium. After 21 days, colonies were stained and colonies > 200 μm diameter were counted in five unslected fields. To assess colony formation at low density, 55 cells per cm² were plated. After 7 days (TERT-NHUC) or 14 days (UM-UC3 and TCCSUP), cells were stained with methylene blue. Total colonies > 30 cells were counted. For determination of resistance to anoikis, cells were seeded on six-well ultra low attachment plates (Corning, Amsterdam, The Netherlands) at 2.6 × 10⁴ cells per cm². After 0, 24 and 48 h, viable cells were quantified using Guava ViaCount and Guava EasyCyte Plus Flow Cytometry System (Millipore).

## RESULTS

**Necdin is expressed in normal human tissues but downregulated in tumour cell lines.** For the first time, we demonstrated that NDN protein was expressed in multiple tissue types (Figure 1A and Supplementary Table 4). In most tissues examined expression was cytoplasmic. Expression ranged from absent (0) to strong (3; Supplementary Figure 1). Cultured NHUCs expressed NDN transcript and protein. Of 25 tumour cell lines, 22 tumour cell lines exhibited reduced expression of transcript (relative to universal cDNA). Of the five cell lines showing the smallest amount of transcript downregulation (U266, 1847, SHYSYSY, LNCaP and MefJuso), only 1847 cell line demonstrated strong NDN protein expression.

Melanoma cell lines (MMG1, SMYM-PRGP and SKMel5) showed expression greater than universal cDNA (Figure 1B). Expression of NDN protein was detected in MMG1 and SMYM-PRGP but not SKMel5 (Figure 1C). MMG1 and ovarian cell line 1847 showed an additional smaller band reacting with the NDN antibody at approximately 33 kDa. An alternatively spliced isoform, NDN variant bAug10 (NCBI), is described, consisting of 299aa, with a predicted protein mass of 33.8 kDa.

Quantitative real-time PCR was previously performed on UC cell lines (Chapman et al., 2008). Here we performed western blotting on a more extensive panel. Loss of NDN protein expression was seen in 38 out of 44 (86%). Necdin protein was detected only in 253J, CAL-29, 97–24, HT1197, LUC6 and LUC8 (Figure 2A).

**Necdin transcript and protein expression is reduced in UC.** Downregulation of NDN expression compared with NHUC was detected in 35 out of 58 (60%) of UC and NDN transcript levels were not significantly related to stage or grade (Chapman et al., 2008). Here, we stained a panel of UC by IHC. Necdin was scored as 0 to 3. A score of 0 or 1 was considered to be reduced expression (loss) relative to normal urothelium and 2 or 3 as positive. Of 72 (61%) tumours, 44 tumours showed loss of NDN expression. Overall, the frequency of loss of NDN expression was higher in the TMA (86%) than our initial panel.

**Necdin transcript is reduced in multiple types of primary tumours.** Oncomine analysis (http://www.oncomine.org) demonstrated downregulation of NDN transcript in multiple tumour types including bladder (Sanchez-Carbayo et al., 2006), colorectal (Sabates-Bellver et al., 2007; Skrzypczak et al., 2010), ovarian, oesophageal (Kim et al., 2010) and breast (Richardson et al., 2006) compared with the normal tissue (Figure 3). Of note, premalignant tissues (Barrett’s oesophagus, vulva intraepithelial neoplasia) also show downregulation of NDN transcript.

**NDN is hypermethylated in TERT-NHUC, UC cell lines and sequencen.** Epityper MassARRAY methylation analyses of TERT-NHUC showed differential Cpg methylation, validating NDN as a potential epigenetic target (Figure 4A). Infinium HumanMethylation27 BeadChip array analysis was performed on bladder cell lines. Hypermethylation at cg13828758 and cg12532169 best correlated with transcript and protein expression (Table 1) and showed an inverse correlation. Of the 23 out of 45 (51%) cell lines with hypermethylation of cg13828758 and 24 out of 45 (53%) cell lines with hypermethylation of cg12532169 (Figure 4B), only LUC6 expressed NDN protein. LUC6 has trisomy 15 (unpublished data). As NDN is a maternally imprinted gene, it is feasible that LUC6 may have duplicated the silenced allele giving a hypermethylated result, yet still contain an unmethylated paternal allele from which the protein is expressed. 253J (with high NDN expression at the transcript and protein level) had the lowest level of hypermethylation at these probe sites. Methylation array analysis of 40 TaG2 UC, detected hypermethylation at cg13828758 in 8 out of 40 (20%) and at cg12532169 in 22 out of 40 (55%; Figure 4C).

**NDN sequence variants were detected in tumour cell lines.** Sequence variants were identified in 2 of 10 colorectal carcinoma cell lines. c.187 G > A (p.D63N), c.659 G > A (p.R220Q) and c.195C > T (p.G65S) in DLD-1 and c.626C > T (p.A209V) and c.774C > T (p.Y258Y) in HCT116. An additional variant c.488 T > C (p.M163T) was identified in one of five ovarian cell lines examined (1847) (Supplementary Figure 2). 1847 expressed NDN protein, whereas DLD-1 was NDN negative. Missense SNP rs114629863 C/T (p.P88L) was detected in 96-1. Of note, 96-1 lacks NDN transcript or protein expression, yet does not exhibit NDN hypermethylation. Synonymous SNP rs2192206 C/T was detected in 25 out of 77 (32%) of tumour cell lines. As matched normal DNA was not available for the cell lines, we are unable to confirm the somatic origin of these variations.

**Necdin promotes anoikis and represses colony formation and anchorage-independent growth.** Necdin was stably overexpressed
or downregulated by shRNA. Ectopic expression did not affect the rate of proliferation or saturation density of NHUC, TERT-NHUC or UM-UC3 (data not shown). Knockdown of NDN expression in 253J and LUCC6 was confirmed and did not affect the rate of proliferation nor colony formation on plastic at low plating density. However, colony formation at low density was repressed by ectopic expression of NDN in TERT-NHUC and UM-UC3 (Figure 5A). Ectopic expression of NDN in UM-UC3 inhibited anchorage-independent growth (Figure 5B) and promoted anoikis. On average, UM-UC3 cells transduced to re-express NDN, exhibited a 16% decrease in viable cells after 24 h culture relative to empty-vector transduced cells on non-adherent plates (ratio t-test, \( P = 0.05 \); Figure 5B).

**DISCUSSION**

NDN first came to our attention as a gene downregulated following telomerase-mediated immortalisation of NHUC (Chapman et al, 2008). It was downregulated after expression of both hTERT and the non-telomere lengthening variant, hTERT-HA (Counter et al, 1998). Downregulation of NDN is therefore related to telomerase activity rather than telomere-dependent effects. Unlike hTERT, hTERT-HA does not immortalise NHUC, indicating that downregulation of NDN is not sufficient for, or a product of immortalisation.

In contrast to the restricted expression of NDN described in the mouse (Maruyama et al, 1991), NDN transcript is detected in various human tissues (Jay et al, 1997), although protein expression had not been previously examined. For the first time, we have shown that NDN is expressed at the protein level in a range of human tissues including bladder, brain, colon and liver. These preliminary studies were limited by the low number of specimens from each normal tissue examined. Further investigation involving multiple normal tissue specimens will be required.
Necdin protein was absent from most cancer cell lines investigated but as described for other transcripts in other tumour cell lines and tumours (Stark et al., 2006), a direct correlation between protein and transcript levels was not observed. Post transcriptional or post-translational modifications are likely to impact on protein expression (de Sousa Abreu et al., 2009). The anti-NDN antibody detected an additional smaller protein in three cell lines, which may be NDN variant bAug10. The functional significance of this and whether this is a tumour-specific variant are unknown.

Our preliminary data suggest that further investigation is warranted in bladder, colorectal, ovarian and brain cell lines and tumours. Indeed, oncomine analysis (Rhodes et al., 2007) demonstrates reduced expression of NDN transcript in multiple tumour types (e.g., bladder, breast, ovarian and colorectal cancer) compared with normal tissue. Downregulation of expression was also detected in two types of premalignant lesions (vulval intraepithelial neoplasia and Barrett’s oesophagus), supporting our previous hypothesis that the changes in gene expression identified in our study of telomerase-immortalised cells may be early events in tumorigenesis. Telomerase activation or over-expression of its RNA components is detected in multiple premalignant lesions including those that can lead to the

Figure 3. Oncomine analysis of deposited expression microarray data shows downregulation of NDN transcript in multiple tumour types compared with normal tissue.
development of bladder, colon, lung, breast, oral and cervical cancer (Engelhardt et al., 1997; Kolquist et al., 1998; Leuenroth et al., 2005; Lantuejoul et al., 2007). ‘Telomerase-associated’ changes in gene expression such as those identified as ‘signature genes’ in TERT-NHUC may also occur in these premalignant lesions and may represent one step during tumour pathogenesis. The possibility that these changes are epigenetically mediated rather than due to genetic deletion, opens up the potential for the use of demethylating or telomerase-inhibiting drugs as a tumour preventative strategy. The detection of telomerase-associated hypermethylation may also have

Figure 4. (A) Output from Sequenom MassArray Epityper analysis for of NDN. Chart shows the average level of methylation in NHUC and TERT-NHUC at each interrogated CpG site. Triangles represent the position of probes in the Human Methylation27 array. From left to right probes are: cg01989224, cg18552939, cg13828758, cg12532169, cg18602919 and cg06743811. (B) Unsupervised cluster analysis of methylation levels (β-values) detected in UC cell lines and (C) primary tumours by methylation array analysis. T represents tumour and N represents normal urothelium. Methylation level is shown from β-value of 0 (red) to 1 (green).
application in early disease detection or monitoring. Further studies are underway to investigate NDN expression and hypermethylation in premalignant tissues.

Our initial focus was on UC and we have shown that NDN is expressed in normal urothelium but expression is lost in the majority of UC. NDN is maternally imprinted and therefore

| Cell line | Transcript | Protein | cg06743811 | cg18602919 | cg12532169 | cg13828758 | cg18552939 | cg01989224 |
|-----------|------------|---------|------------|------------|------------|------------|------------|------------|
| 253J      | +          | +       | +          | +          | -          | -          | -          | +          |
| HT1197    | +          | +       | -          | -          | +          | -          | -          | -          |
| HT1376    | -          | -       | -          | -          | +          | -          | -          | -          |
| LUC1      | -          | -       | -          | -          | -          | -          | -          | -          |
| LUC2      | -          | -       | +          | -          | +          | -          | -          | -          |
| LUC3      | -          | -       | -          | -          | -          | -          | -          | -          |
| LUC4      | -          | -       | +          | -          | +          | -          | -          | -          |
| LUC5      | -          | -       | -          | -          | -          | -          | -          | -          |
| LUC6      | +          | +       | +          | -          | +          | +          | +          | +          |
| LUC7      | -          | -       | -          | +          | +          | -          | -          | -          |
| LUC8      | -          | -       | -          | +          | -          | -          | -          | -          |
| UBLCl     | -          | -       | -          | -          | -          | -          | -          | -          |
| SW780     | -          | -       | -          | -          | -          | -          | -          | -          |
| 97-24     | +          | +       | -          | +          | -          | -          | -          | -          |
| 96-1      | -          | -       | -          | -          | -          | -          | -          | -          |
| 97-7      | -          | -       | -          | -          | -          | -          | -          | -          |
| 97-1      | -          | -       | -          | -          | +          | +          | +          | -          |
| 92-1      | -          | -       | -          | +          | +          | -          | -          | -          |
| 97-18     | -          | -       | -          | +          | +          | -          | -          | -          |
| 94-10     | -          | -       | -          | +          | +          | -          | -          | -          |
| KU-19-19  | -          | -       | -          | -          | -          | -          | -          | -          |
| TCCSUP    | -          | -       | -          | -          | -          | -          | -          | -          |
| 647V      | -          | -       | -          | -          | -          | -          | -          | -          |
| JMSU1     | -          | -       | +          | -          | -          | -          | -          | -          |
| RT112M    | -          | -       | -          | -          | -          | -          | -          | -          |
| CAL29     | +          | +       | -          | -          | -          | -          | -          | -          |
| VM-CUB-II | -          | -       | -          | -          | -          | -          | -          | -          |
| MGH – U3  | -          | -       | -          | -          | -          | -          | -          | -          |
| DSH1      | -          | -       | +          | -          | +          | -          | -          | -          |
| SW1710    | -          | -       | -          | +          | +          | -          | -          | -          |
| VM-CUB-III| -          | -       | -          | +          | +          | -          | -          | -          |
| T24       | -          | -       | -          | -          | +          | -          | -          | -          |
| BFTC909   | -          | -       | +          | +          | +          | -          | -          | -          |
| BFTC905   | -          | -       | -          | +          | +          | -          | -          | -          |
| 5637cl1   | -          | -       | -          | +          | +          | -          | -          | -          |
| BC-3C     | -          | -       | -          | +          | +          | -          | -          | -          |
| SD        | -          | -       | -          | +          | +          | -          | -          | -          |
| VM-CUB-I  | -          | -       | -          | +          | +          | -          | -          | -          |
| UM-UC3    | -          | -       | -          | +          | +          | -          | -          | -          |
| SCaBER    | -          | -       | +          | +          | -          | -          | -          | -          |
| 639V      | -          | -       | +          | +          | +          | -          | -          | -          |
| RT4       | -          | -       | +          | +          | +          | -          | -          | -          |
| J’ON      | -          | -       | -          | -          | -          | -          | -          | -          |
| J82       | -          | -       | -          | -          | -          | -          | -          | -          |
| HCV29     | -          | -       | -          | +          | +          | -          | -          | -          |

Abbreviation: NDN = necdin.
Necdin is hypermethylated and downregulated in cancer

Alterations in methylation status are therefore only detected in the tumour cell lines. Perhaps not unexpectedly for an imprinted locus has the technical difficulty that methylation levels in control normal samples have -values of approximately 0.5 (0.474 measured here for cg12532169 and 0.583 for cg13828758). Alterations in methylation status are therefore only detected in the range 0.5–1.0, half the range that could be measured in a non-imprinted gene. We chose a robust cutoff of a 20% increase in methylation level compared with control sample and this may have been over conservative in this context and led to under reporting of hypermethylation.

Mutation screening was performed for the single exon of NDN in tumour cell lines. Perhaps not unexpectedly for an imprinted gene with such frequent hypermethylation, mutations were not observed in UC cell lines. However, mutations were identified in DLD-1 and HCT116 colon carcinoma cell lines and 1847 ovarian carcinoma cell line. The mutations occur in regions of high evolutionary conservation and we predict that they could be of functional significance. The functional domains of NDN necessary for protein–protein interaction, nuclear matrix targeting and cell growth suppression have been identified (Taniura et al, 2005). The whole MAGE homology domain (amino acids 116–280) is required for interaction with p53 and could be disrupted by p.R220Q, p.M163T or p.A209V. p.R220Q and p.A209V are within the part of the domain required for cell growth suppression (amino acids 191–222). p.M163T is within domains required for nuclear matrix targeting (amino acids 144–184). No targeted mutation screen of NDN has previously been performed in human cancer. However, the Catalogue of Somatic Mutations in Cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/) at the time of writing, lists 43 mutations of NDN found during genome-wide mutation screens. These include 28 in lung tumours plus 5 examples in colorectal tumours. Mutations are also reported in skin squamous cell carcinoma (p.P309L), ovarian serous carcinoma (p.P41S), breast carcinoma (E269K, R142H), kidney (P34P, H161H), prostate (P88T) and endometrial cancer (G130S, R142C, R249C). This indicates that mutation screening of non-UC tumour types, particularly lung and colorectal cancers, would be worthwhile. The missense SNP rs114629863 C/T (p.P88L) was detected in a UC cell line, 96-1. This change is within the MAGE homology domain required for p53 interaction and therefore potentially could impact on protein function (Taniura et al, 2005). Interestingly, 96-1 is a cell line that lacks NDN transcript or protein and does not exhibit NDN hypermethylation. We investigated the functional significance of NDN silencing. Necdin is a suppressor of proliferation in post mitotic neurons (Taniura et al, 1998). Here, no effect of NDN on NHUC or tumour cell line proliferation was observed. Ectopic expression of NDN suppresses colony formation and growth in SAOS-2 osteosarcoma cells (Taniura et al, 2005). Necdin was ectopically overexpressed in cell lines that had low or minimal

Figure 5. Phenotypic effect of NDN overexpression. (A) Colony formation at low plating density in TERT-NHUC and UM-UC3. Photo shows 9 cm diameter dishes stained with methylene blue. Top row, UM-UC3-empty vector, bottom row NDN-transduced UM-UC3. (B) Anchorage-independent growth of UC cell line, UM-UC3. *Indicates a statistical significant difference between empty vector and NDN-transduced cells. Data are representative of at least triplicate experiments. Error bars represent standard deviations of multiple samples.
endogenous levels and reduced colony formation at low density was observed in TERT-NHUC, UM-UC3 and TCCSUP. Reduced anchorage-independent growth was observed in UM-UC3. Furthermore, anoikis was promoted in UM-UC3, which may have contributed to the repression of anchorage-independent growth. In light of our current evidence supporting the idea of NDN as a tumour suppressor gene, future studies to investigate the effect of NDN on tumorigenesis in vivo may now be warranted. Indeed, ectopic overexpression of NDN in a mouse tumour cell line is reported to attenuate tumorigenicity and metastasis in vivo. The same study found that the gene expression signature that results from ectopic overexpression of NDN is also reported to predict prognosis in a human breast cancer cohort (Crawford et al, 2008). There is considerable evidence to support the hypothesis that NDN is a tumour suppressor gene (Chapman and Knowles, 2009).

Necdin is one of the genes inactivated in PWS. If NDN is proven to be a tumour suppressor gene, this raises the question of whether PWS patients have an increased cancer risk. Although it is not a recognised cancer-prone syndrome, the only specific study of cancer incidence in PWS patients reported an increased risk of myeloid leukaemia but not of other cancers (Davies et al, 2003). The report by Liu et al (2009) describing the importance of NDN in maintaining hematopoietic stem cell quiescence perhaps provides evidence to explain this observation. Prader–Willi syndrome has only been accurately diagnosed in the last 30 years since genetic testing became possible. Therefore, there are only few confirmed cases identified in middle aged or elderly patients, the population in which a general increased cancer predisposition would present (Davies et al, 2003). As life expectancy for well-managed PWS is now near normal, any increased cancer predisposition may be observed in the future. However, the concept of a gene whose germ line alteration results in a syndrome, which is not tumour prone, whereas a somatic alteration contributes to tumorigenesis is not unprecedented. Other examples include FGFR2 and FGFR3 where germ line activating mutations are implicated in craniostenosis and chondrodysplasia syndromes but somatic mutations contribute to endometrial (Pollock et al, 2007) and urothelial (van Rhijn et al, 2002) cancers. In conclusion, we have shown that NDN is widely expressed in normal human tissue. Expression is lost in cell lines derived from a range of common tumour types. NDN is verified as an epigenetic signature that results from ectopic overexpression of NDN and this correlates with loss of expression in UC cell lines. Furthermore, unmethylation of NDN is a novel candidate tumour suppressor gene with relevance to a wide range of human cancers.

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