ARID1B, a member of the human SWI/SNF chromatin remodeling complex, exhibits tumour-suppressor activities in pancreatic cancer cell lines

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Background: The human ATP-dependent SWItch/sucrose nonfermentable (SWI/SNF) complex functions as a primary chromatin remodeler during ontogeny, as well as in adult life. Several components of the complex have been suggested to function as important regulators of tumorigenesis in various cancers. In the current study, we have characterised a possible tumour suppressor role for the largest subunit of the complex, namely the AT-rich interaction domain 1B (ARID1B).

Methods: We performed Azacytidine and Trichostatin A treatments, followed by bisulphite sequencing to determine the possible DNA methylation-induced transcription repression of the gene in pancreatic cancer (PaCa) cell lines. Functional characterisation of effect of ARID1B ectopic expression in MiaPaCa2 PaCa cell line, which harboured ARID1B homozygous deletion, was carried out. Finally, we evaluated ARID1B protein expression in pancreatic tumour samples using immunohistochemistry on a tissue microarray.

Results: ARID1B was transcriptionally repressed due to promoter hypermethylation, and ectopic expression severely compromised the ability of MiaPaCa2 cells to form colonies in liquid culture and soft agar. In addition, ARID1B exhibited significantly reduced/loss of expression in PaCa tissue, especially in samples from advanced-stage tumours, when compared with normal pancreas.

Conclusion: The results therefore suggest a possible tumour-suppressor function for ARID1B in PaCa, thus adding to the growing list of SWI/SNF components with a similar function. Given the urgent need to design efficient targeted therapies for PaCa, our study assumes significance.

Pancreatic cancer (PaCa) continues to be a devastating disease despite recent improvements in the understanding of its biology. Pancreatic tumours are frequently associated with post-resection recurrence and are refractory to available treatment options (Buchler et al, 1991). Most patients harbour distant metastasis at the time of presentation, and therefore not eligible for resection; the 5-year survival rate for such patients is close to nil. Identification of PaCa oncogenes exhibiting recurrent activation including KRAS and AKT2, and of tumour suppressor genes (TSGs) exhibiting recurrent inactivation including TP53, CDKN2A

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and SMAD4 has improved our understanding of pancreatic tumorigenesis (Bardeesy and DePinho, 2002). Recent advances in the field of cancer genomics have revealed interesting insights into the complex biology of the disease and are expected to hasten the development of efficient targeted therapies. Given the ease of targeting, researchers worldwide have focused their efforts on characterisation of PaCa oncogenes; the study of PaCa TSGs has been neglected to a significant extent. Of note, recent studies have indicated that a majority of driver mutations in all cancers occur in TSGs rather than oncogenes (Bozic et al, 2010).

Epigenetic modifications are cardinal regulators of eukaryotic gene expression, especially with respect to embryonic development and oncogenic transformation. The human SWItch/sucrose nonfermentable (SWI/SNF) ATP-dependent chromatin-remodelling complex, components of which are orthologs of the Drosophila trithorax family (Kennison, 1995; Schuettengruber et al, 2007), includes either of the two ATPase-containing subunits, namely BRG1 and BRM. The largest subunit named BAF250 or AT-rich includes either of the two ATPase-containing subunits, namely harbours BRG1 another variant (the PBAF complex) has been reported, which subunits, four distinct complexes can be envisaged. In addition, another variant (the PBAF complex) has been reported, which harbours BRG1 in association with BAF180 and BAF200 (Yan et al, 2005). Mutation-deletion-inactivated inactivation of BRG1 and, to a lesser extent, of BRM has been described in many tumour types (Weissman and Knudsen, 2009). Recently, inactivation of SNF5 (a core component of the complex), resulting in alleviation of repression of Polycomb group proteins such as EZH2, was shown to be an important driver event in malignant rhabdoid tumours (Wilson et al, 2010). In addition, tumour-suppressor roles for BAF155 and BAF57 (core components of the complex) have also been validated (Weissman and Knudsen, 2009).

Our earlier studies based on array-based comparative genomic hybridisation (aCGH) carried out on PaCa cell lines (Bashyam et al, 2005) and xenografts (Kwei et al, 2008) led to the identification of several localised amplifications and deletions in specific chromosomal regions. Characterisation of two recurrent amplifications at 18q11.2 and 7q21 led to the assignment of an oncogenic function to GATA6 (Kwei et al, 2008) and SMURF1 (Kwei et al, 2011), respectively, in PaCa. More importantly, we identified several localised homozygous deletions validated by multiplex PCR, including one located at 6q25.3 that harboured a single annotated gene viz ARID1B (Bashyam et al, 2005). In the current study, we propose a tumour-suppressor function for ARID1B in PaCa based on functional studies, methylation analysis of promoter CpG islands and expression analysis in pancreas tumour samples.

MATERIALS AND METHODS

PaCa cell line propagation and manipulation. PaCa cell lines were procured from the ATCC, USA; propagation and DNA/RNA isolations were performed as described earlier (Bashyam et al, 2005). For Azacytidine and Trichostatin A (TSA) (Sigma, St. Louis, MO, USA) treatments, 0.2 million cells were seeded in 60-mm dishes and exposed (amounts (in µM)) indicated in Figures 1A and B) for 24 h, followed by a fresh exposure for 24 h. Quantitative reverse transcription PCR was performed to evaluate ARID1B transcript levels relative to GAPDH as described in Supplementary Methods S1.

Analysis of ARID1B CpG methylation. Putative CpG island was identified upstream of the ARID1B start codon using MethPrimer (www.urogene.org/methprimer/index1.html) and CGPLOT (www.ebi.ac.uk/Tools/emboss/cgplot) with default parameters.

PCR primer pairs specific to modified DNA sequence and flanking the putative ARID1B CpG-rich sequence were designed using MethPrimer such that each amplicon size was < 400 bp (Figure 1C top panel and Supplementary Methods S1). All primers (except 3PR) did not include CpG dinucleotide (sequences are listed in Supplementary Methods S1). Bisulphite modification of genomic DNA isolated from cell lines was performed using EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA), as per manufacturer’s instructions. PCR products were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA), as per standard protocol. Recombinant plasmids were sequenced using the 3130 × 1 genetic analyser (ABI Inc, Foster city, CA, USA), as per the manufacturer’s instructions. A conversion frequency of > 98% was confirmed in each recombinant plasmid.

Ectopic expression of ARID1B and functional assays. ARID1B cDNA construct (KIAA1235), a kind gift from the Kazusa DNA research institute (Chiba, Japan), encodes a partially truncated 1485 amino acid protein (devoid of the N-terminal 805 amino acids). Previous studies have validated functional activity of the ARID1B protein expressed from this construct (Inoue et al, 2002, 2011). ARID1B cDNA was cloned into the pcDNA3.1HisC vector as described in Supplementary Methods S1. MiaPaCa2 cells were transfected with the ARID1B-pcDNA3.1HisC recombinant plasmid construct in 60-mm culture dishes using lipofectamine 2000 (Invitrogen), as per manufacturer’s protocol; pcDNA3.1HisC vector was transfected separately as a control. Transfected cells were passaged into 100-mm dishes in presence of 100 µg ml⁻¹ Neomycin (G418) to obtain a single cell spread. Neomycin-resistant colonies appeared after 20–21 days of selection and were isolated by localised trypsinization method, grown separately on 96-well plates and further expanded in 6-well plates. Expression of the Neomycin gene in recombinant clones was confirmed by RT–PCR (data not shown). ARID1B expression was confirmed using RT–PCR in the ARID1B clones (but not in the vector clones), as per strategy outlined in Supplementary Methods S1. In separate transfection experiments, colonies (obtained from both ARID1B and vector transfecants of MiaPaCa2 and Panc1 cells) were pooled independently following 21 days of selection under Neomycin to perform a colony-formation assay (Supplementary Methods S1). Other functional assays were performed as described in Supplementary Methods S1.

Construction of tissue microarray (TMA) and immunohistochemistry (IHC). A PaCa TMA was constructed using the Minicore tissue arrayer (Alphelys, Plaisir, France) as described previously (Ginestier et al, 2002). A total of 52 sample pairs (tumour and matched normal) in the form of formalin-fixed paraffin-embedded blocks representing pancreatic tumours were collected from the Nizam’s Institute of Medical Sciences, Hyderabad, following approval from the hospital ethics committee. The samples included well, moderate and poorly differentiated ductal adenocarcinoma; specialized adenocarcinoma including cyst-adeno-carcinoma, mucinous and papillary adenocarcinoma and clear cell carcinoma; and neuroendocrine tumours. The array was constructed with at least two representative 1-mm cores from each tumour and normal sample. IHC was performed on 4-µm sections on an automated immunostainer (Ventana Benchmark XT, Tucson, AZ, USA) using ARID1B (clone 2F2, Novus Biologicals, Littleton, CO, USA; dilution of 1 : 200) and p53 (clone DO-1, EMD Millipore Calbiochem, Billerica, MA, USA; dilution of 1 : 100) antibodies separately, as per manufacturer’s instructions. For ARID1B, nuclear staining intensity (absent, weak, moderate and strong: 0–3 scale) and fractional epithelium staining (absent, up to 25, 50, 75 and 100%; 0–4 scale) were evaluated by the pathologist and summated for a final staining score. For p53, >20% positivity was considered as nuclear stabilisation.
RESULTS

ARID1B exhibits promoter CpG methylation-induced transcriptional repression in PaCa cell lines. TSGs are inactivated in tumour cells through several distinct mechanism(s), not the least being mutation and methylation-induced transcription silencing. We screened genomic DNA isolated from 10 PaCa cell lines for presence of mutations in the coding region, promoter, UTRs and splice consensus sequences by PCR-DNA sequencing and did not detect a single mutation (data not shown). In addition, the same ARID1B transcript isoform was detected in PaCa cell lines and pancreatic normal tissue (data not shown). Azacytidine, frequently used to determine genes transcriptionally repressed through methylated CpGs in the promoter, caused a significant elevation in ARID1B transcript levels in SW1990, PANC10.05 and CFPAC1, but not in PANC8.13, HPAFII and PANC1, commensurate with lower (SW1990, PANC10.05 and CFPAC1; Supplementary Figure S1) and higher (PANC8.13, HPAFII and PANC1; Supplementary Figure S1) ARID1B transcript levels (Figure 1A).

In addition, Azacytidine had no significant effect on ARID1B transcript level in the nontumorigenic human pancreatic ductal epithelial (HPDE) cells (Figure 1A) known to exhibit adequate expression of SWI/SNF components, including ARID1B (Ouyang et al, 2000; Shain et al, 2012). Elevation in ARID1B transcript levels was evident in SW1990, PANC10.05 and CFPAC1 when subjected to TSA treatment as well (Figure 1B).

Next, we identified CpG-rich sequences in the putative promoter region of ARID1B, spanning about 2 kb upstream of the translation initiation codon (Figure 1C top panel). Bisulphite sequencing using primer pairs specific for the CpG-rich regions (Figure 1C and Supplementary Methods S1) revealed significant methylation of CpGs located in the P3 region (present immediately upstream to the ARID1B translation initiation codon; Figure 1C top panel), particularly in SW1990. Further, the methylation (in SW1990) was completely alleviated upon Azacytidine treatment (Figure 1C, bottom panel), suggesting that methylation was responsible for ARID1B transcription silencing. Moderately significant methylation in P2 and P4 regions was also observed (Supplementary Figure S2), though no significant methylation was
detected in the P1 region (data not shown). In contrast, PANC8.13, HPAFII and HPDE either harboured negligible or nil methylation of the CpGs in these regions (Figure 1C and Supplementary Figure S2).

Ectopic expression of ARID1B abrogates tumour-related characteristics of PaCa cell line. In our previous study, the 6q25.3 homozygous deletion (harbouring the ARID1B gene) identified in the MiaPaCa2 PaCa cell line was PCR validated using primer pairs specific for the ARID1B 3'-UTR region (Bashyam et al., 2005). Therefore, we first confirmed ARID1B complete deletion using PCR primers specific for exons 2, 5, 10, 12, 15, 17 and 20 (data not shown). Next, in order to determine the effect of ARID1B ectopic expression in MiaPaCa2 cells, we generated pcDNA3.1HisC clones of ARID1B (and of the vector as a control) as described in the MATERIALS AND METHODS section. We chose two vector and ARID1B clones each for analysis. Expression of ARID1B transcript in the ARID1B clones was confirmed using RT-PCR (Supplementary Figure S3); however, protein expression could not be detected, though three different antibodies were tested (data not shown). MTT assay (Figure 2A), crystal violet dye extraction growth assay (Figure 2B), a standard plating assay (percentage of cells adhering to the surface upon seeding; data not shown) as well as FACS-based cell cycle analysis (data not shown) did not reveal a significant difference in growth kinetics of ARID1B and vector clones. Similarly, the percentage of apoptotic population in the gene and vector clones was not significantly different (data not shown). However, the ARID1B clones exhibited significantly reduced ability to form colonies in liquid culture as compared with the vector clones (Figures 2C and D), indicating a reduced clonogenicity in case of the former. In addition, the ARID1B clones generated significantly
less number (and smaller size) of colonies in soft agar, indicating a reduced ability to grow in the absence of anchorage (Figures 2E and F). In order to rule out the possibility of reduced colony-formation ability of ARID1B clones being attributable specifically to the two selected clones and not to ARID1B expression, we performed liquid colony-formation assay separately using a ‘pool’ of ARID1B and vector clones obtained from an independent MiaPaCa2 transfection experiment (MATERIALS AND METHODS). The pooled ARID1B clones generated significantly fewer colonies compared with the pooled vector clones, whereas there was no significant difference in colonies generated from pooled vector and ARID1B PANC1 clones (Supplementary Figure S4), thus strengthening our premise that ARID1B expression was indeed responsible for reduced colony-formation ability. Given that ectopic expression of the SWI/SNF component BRG1 induces a senescence phenotype in rat mesenchymal stem cells (Napolitano et al, 2007) as well as in PaCa cells (Shain et al, 2012), we tested whether ARID1B expression could have a similar effect on MiaPaCa2 cells. The ARID1B clones exhibited significantly higher senescence-associated β-galactosidase activity when compared with the vector clones (Supplementary Figure S5 and Figure 2G).

Reduced/loss of ARID1B expression in PaCa tissues.

We constructed a TMA to evaluate ARID1B and p53 expression status in PaCa tumour samples using IHC (Figures 3A and B). Interestingly, a significant proportion of tumour samples exhibited negative/reduced staining when compared with normal pancreas (P = 0.0001, Fisher’s exact test; Figure 3C). Moreover, the loss of ARID1B expression was significantly associated with advanced-tumour stage (P = 0.0185, Fisher’s exact test; Figure 3D), indicating perhaps it could be a late event. As a comparison, p53 exhibited nuclear stabilisation in 19 of 42 samples (representative result shown in Figure 3B) and there was no association with ARID1B status nor with tumour stage (data not shown). These results provide further evidence for a tumour-suppressor role for ARID1B in PaCa.

**DISCUSSION**

Deletion or rearrangement of the 6q25.3 region has been described in breast (Utada et al, 2000), cervical (Mazurenko et al, 2003) and
other cancers (Monoranu et al, 2008). In the current study, we endeavoured to characterise a homozygous deletion localised at 5q25.3, identified in the MiaPaCa2 PaCa cell line in our previous studies (Bashyam et al, 2005; Shain et al, 2012) and in other studies (Birnbaum et al, 2011), which included only one annotated gene viz ARID1B. Gene mutation is a common mode of inactivation of TSGs; however, we did not detect a single mutation in 10 PaCa cell lines tested. Azacytidine (Figure 1A) and Trichostatin A (Figure 1B) treatments, however, resulted in significant elevation in ARID1B transcript level in PaCa cell lines, suggesting suppression of gene expression through DNA methylation, which was confirmed by bisulphite sequencing (Figure 1C). HLTF, encoding another member of the SWI/SNF family, was earlier shown to be transcriptionally repressed following CpG methylation in lung cancer cell lines, and the repression was alleviated upon treatment with Azacytidine (Moinova et al, 2002).

Ectopic expression of SWI/SNF components into cancer cells has been shown to result in elevation of antitumor characteristics such as cell cycle arrest, apoptosis or differentiation, depending on the cancer cell type and the identity of the inactivated component (Caramel et al, 2008; Dunaief et al, 1994; Wang et al, 2005). Recent studies have suggested a tumour-suppressor role for components of the SWI/SNF complex in several cancers (Wilson and Roberts, 2011). siRNA-mediated knockdown of ARID1B was earlier shown to cause a significant reduction of growth-factor-independent growth in HPDE (Shain et al, 2012), further corroborating our observations. The reduced/loss of ARID1B expression in PaCa when compared with normal pancreatic tissues (Figure 3C) suggests the possible importance of the gene in human pancreatic tumours.

Previous studies on preosteoblast cell lines revealed opposing roles for ARID1A and ARID1B-containing SWI/SNF complexes, with the former causing cell cycle arrest, whereas the latter appeared to be involved in cell cycle progression (Nagl et al, 2007). On the other hand, ARID1B levels increase during differentiation of embryonic stem cells (Kaeser et al, 2008) and ectopic expression induces increased expression of p53 and p21, followed by cell cycle arrest in HeLa cells (Inoue et al, 2011). siRNA-mediated knockdown of ARID1B was earlier shown to cause a significant reduction of growth-factor-independent growth in HPDE (Shain et al, 2012), further corroborating our observations. The reduced/loss of ARID1B expression in PaCa when compared with normal pancreatic tissues (Figure 3C) suggests the possible importance of the gene in human pancreatic tumours.

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