Oncogene (2008) 27, 3624–3634

www.nature.com/onc

ORIGINAL ARTICLE

Aberrant promoter methylation and tumor suppressive activity of the *DFNA5* gene in colorectal carcinoma

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To identify novel methylated gene promoters, we compared differential RNA expression profiles of colorectal cancer (CRC) cell lines with or without treatment of 5-aza-2′-deoxycytidine (5-aza-dC). Out of 1776 genes that were initially 'absent (that is, silenced)' by gene expression array analysis, we selected 163 genes that were increased after 5-aza-dC treatment in at least two of three CRC cell lines. The microarray results were confirmed by Reverse Transcription–PCR, and CpG island of the gene promoters were amplified and sequenced for examination of cancer-specific methylation. Among the genes identified, the deafness, autosomal dominant 5 gene, *DFNA5*, promoter was found to be methylated in primary tumor tissues with high frequency (65%, 65/100). Quantitative methylation-specific PCR of *DFNA5* clearly discriminated primary CRC tissues from normal colon tissues (3%, 3/100). The mRNA expression of *DFNA5* in four of five colon cancer tissues was significantly downregulated as compared to normal tissues. Moreover, forced expression of full-length *DFNA5* in CRC cell lines markedly decreased the cell growth and colony-forming ability whereas knockdown of *DFNA5* increased cell growth in culture. Our data implicate *DFNA5* as a novel tumor suppressor gene in CRC and a valuable molecular marker for human cancer.
Oncogene (2008) **27,** 3624–3634; doi:10.1038/sj.onc.1211021; published online 28 January 2008

Keywords: promoter methylation; *DFNA5*; colon cancer

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Received 5 September 2007; revised 22 October 2007; accepted 1 December 2007; published online 28 January 2008

Introduction

Aberrant gene expression is a characteristic of human cancers, and changes in DNA methylation status can have profound effects on the expression of genes. Tumor suppressor genes (TSGs) display both genetic and epigenetic inactivation in human tumors, and the transcriptional silencing of TSGs has established hypermethylation as a common mechanism for loss of TSG function in human cancer (Herman, 1999). Thus, knowledge of methylation patterns across the genome can help to identify key TSGs inactivated during tumor formation.

A number of genes are commonly hypermethylated in colorectal cancer (CRC) including *hMLH1*, *p16INK4a*, *p14ARF*, *RAR-β*, *APC*, *MGMT*, *cyclin A1*, *CDX1*, *MYOD1*, *COX-2* and *WT-1* (Esteller *et al*., 2001; Lind *et al*., 2004; Xu *et al*., 2004). Recently, studies also revealed common genetic and/or epigenetic alterations of the *p53* and *p16* tumor suppressor pathways in human CRC (Toyota *et al*., 2000). Since hypermethylated genes may be involved in the etiology of the disease and may be early events in neoplastic progression, these genes represent attractive targets for therapeutic approaches, disease detection and monitoring. However, genes methylated only in neoplastic tissues with high frequency (over 40%) are rare.

The human deafness, autosomal dominant 5 gene (*DFNA5*) at chromosome 7p15, causes an autosomal dominant form of hearing impairment when mutated (van Camp *et al*., 1995). *DFNA5* encodes a protein of 496 amino acids that is found in human cochlea, brain, placenta and kidney cDNA preparations (Van Laer *et al*., 1998). An intronic insertion and/or mutation leads to premature termination of the protein, resulting in a nonsyndromic progressive hearing loss (Van Laer *et al*., 1998). *DFNA5* was also designated *ICERE-1* (inversely correlated with estrogen receptor expression) due to its lower expression in estrogen receptor (*ER*)-positive breast cancers compared to *ER*-negative tumors (Thompson and Weigel, 1998). In addition, etoposide resistance in melanoma cells is associated with decreased *DFNA5* expression (Lage *et al*., 2001). Increased expression of *DFNA5* in these cells confers

elevated cellular susceptibility to trigger a caspase-3-dependent apoptotic signal pathway after etoposide exposure. These results suggest that the *DFNA5* gene product may participate in melanoma and breast cancer progression and perhaps resistance to chemotherapy.

A combination of pharmacological unmasking and oligonucleotide microarray analysis (Yamashita *et al*., 2002; Kim *et al*., 2006) enabled us to find novel methylated genes in CRC cell lines and primary CRC tissues. Among the new genes identified, *DFNA5* exhibited epigenetic activation at high frequency. Moreover, functional studies suggested a tumor suppressive role for DFNA5 in CRC cells.

Results

Pharmacological unmasking and subsequent differential microarray analysis in CRC cell lines

We used the demethylating agent 5-aza-2′-deoxycytidine (5-aza-dC) to reactivate genes epigenetically silenced in three CRC cell lines (HCT116, HT29 and DLD-1), and performed a survey of tumor suppressor gene candidates using microarray chips containing 22 284 transcripts (Affymetrix, Santa Clara, CA, USA). Complete silencing of expression is characteristic of methylated genes (Yamashita *et al*., 2002; Kim *et al*., 2006); we therefore selected 1776 genes showing 'absent' expression in all three cell lines before pharmacological treatment (Figure 1 and Supplementary Table 1). The 1776 genes may be frequently inactivated in colon cancer, and candidate genes regulated by DNA promoter methylation may be reactivated by 5-aza-dC treatment. Thus, we excluded genes that were 'no change' after pharmacological treatment in three cell lines (528), and remaining 1248 genes were 'increased' or 'marginally increased' by the 5-aza-dC treatment in one of three cell lines. We further reasoned that commonly reactivated genes after pharmacological unmasking in at least two of three CRC cell lines were more likely to represent frequently inactivated TSGs in colon cancer (163 genes). Among them, 21 genes were increased by the 5-aza-dC treatment in all the three CRC cells lines tested. These genes included cancer/testis antigens (*GAGE 2, 3, 4, 7B*) and lymphocyte antigen 6 (*E48*) that were 'absent' in the absence of and 'increased' in the presence of 5-aza-dC treatment. We further diminished the number of candidate genes by ruling out genes of which methylation status was already known (For example, NY-ESO-1, ERCC, APOC-1, DAPK-1, SPARC, and stratifin), those with a basal expression in the microarray that was called 'absent' but the hybridization intensity (fluorescence signal in the microarray) was still relatively high (>50). Basal expression (before treatment with 5-aza-dC) of most genes with hybridization intensity over 50 in the microarray was confirmed in cell lines by reverse transcription (RT)–PCR analysis. Genes that had no CpG island in their promoters proximal to the transcriptional start site (TSS) were eliminated leaving 30 genes. These 30 genes were thus categorized as likely candidates for high frequency methylation.

Expression and promoter hypermethylation in CRC cell lines

We then examined the expression of the 30 genes by RT–PCR. We found that about 50% of these genes had

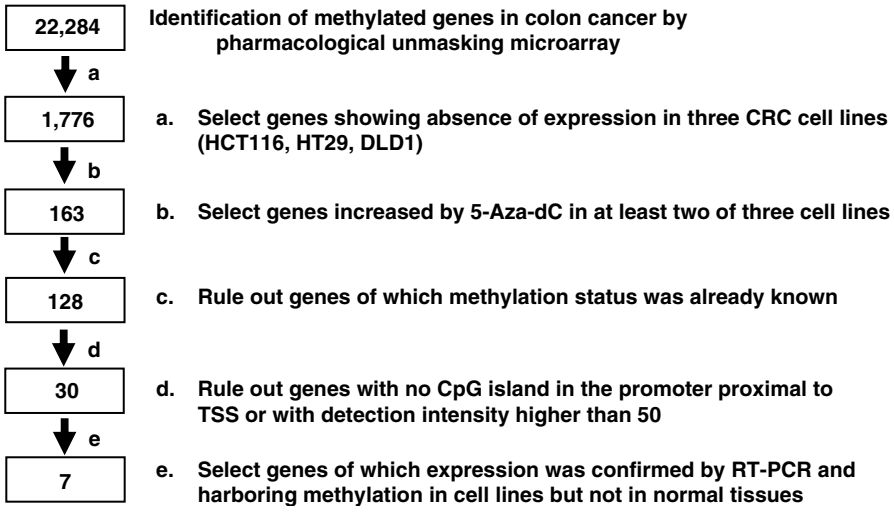

Figure 1 Flowchart for selection of candidate genes. We used three colorectal cancer (CRC) cell lines to screen for candidate tumor suppressor genes (TSGs) after 5 μM 5-aza-2′-deoxycytidine (5-aza-dC) treatment followed by cRNA hybridization to a 22 284-oligonucleotide microarray. We obtained 1776 candidates, which showed absence of expression in any CRC cells before treatment. We diminished the number of candidates by selecting genes commonly increased by 5-aza-dC (increased in two of three cell lines; 163). We further removed genes of which methylation status was already known, genes with relatively high hybridization intensity or genes with no CpG island in the promoters (30). We selected 21 genes to examine for methylation analysis by direct sequencing, and finally selected 7 genes which exhibited a profile of complete absence in three CRC cell lines and common reactivation after the demethylation treatment in reverse transcription (RT)–PCR analysis. These genes harbored promoter methylation in cell lines but not in normal tissues.

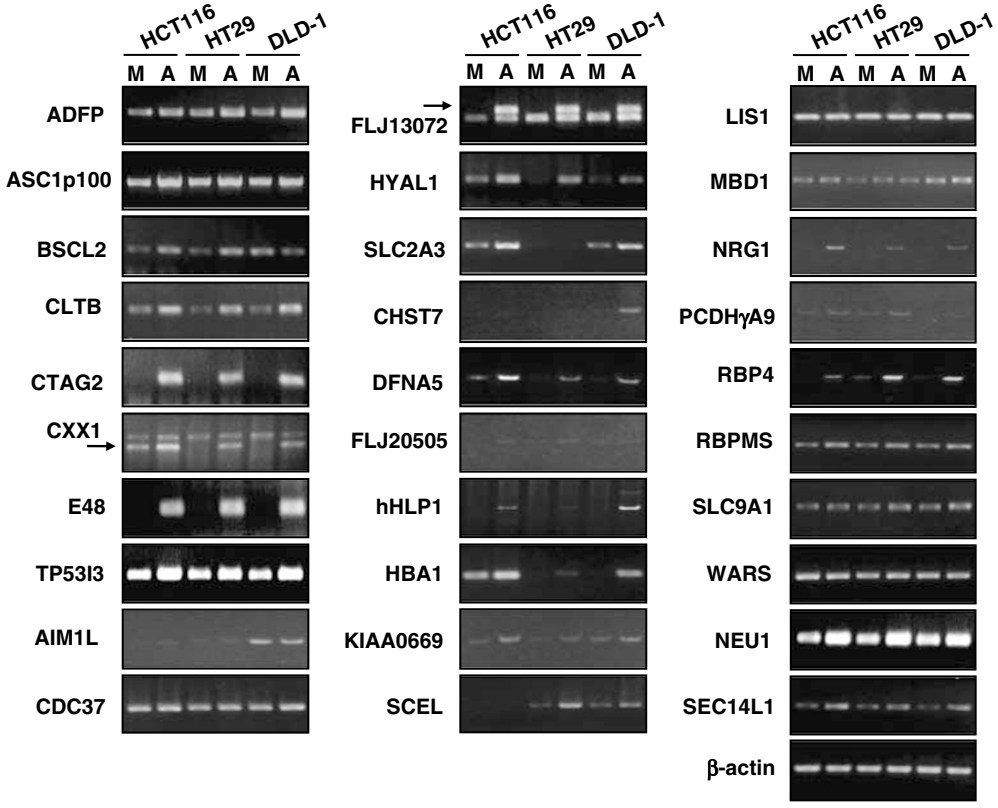

Figure 2 Candidate gene expression in colorectal cancer (CRC) cell lines by reverse transcription (RT)–PCR. RT–PCR was performed with the same RNA samples used for microarray analysis after cDNA synthesis. β-Actin expression is shown as a loading control. Two close PCR bands at *CXX1* and *FLJ13072* were separately excised, gel-extracted and sequenced. Lower band of *CXX1* and upper band of *FLJ13072* were the PCR products of each gene amplification (arrows). Some genes were not silenced in CRC cell lines nor reactivated by 5-aza-dC treatment. Cancer/testis antigen 2 (*CTAG2*) and lymphocyte antigen 6 (*E48*) exhibit tissue-restricted expression (Brakenhoff *et al*., 1997, Scanlan *et al*., 2004). m (mock), no treatment; a, 5-aza-dC treatment.

still detectable level of transcript in CRC cell lines (Figure 2). Most of genes (27/30, 90%) were weakly or significantly upregulated after 5-aza-dC treatment (Figure 2 and Supplementary Table 2). The basal expression of *CTAG2* (cancer/testis antigen 2), *CXX1* (putatively prenylated protein), *DFNA5*, *E48* (E48 antigen), *FLJ13072*, *HBA1* (hemoglobin alpha-1 globin chain), *hHLP1* (HAP1-like protein), *NRG1* (neuregulin 1) and *RBP4* (retinol binding protein 4), was undetectable in two of three CRC cell lines. *CXX1*, *DFNA5* and *HBA1* were weakly expressed only in HCT116, and *RBP4* was weakly expressed only in HT29. The remaining five genes were completely silenced in all three CRC cell lines tested.

To investigate promoter methylation of these candidate genes, we analysed their gene promoters in all three CRC cell lines by bisulfite sequencing, and found that 10 genes showed methylation in at least one of three CRC cancer cell lines (Table 1). We also examined the promoter methylation status in four normal colon mucosa samples in order to rule out genes, also methylated in normal tissues. *ADFP* (adipose differentiation-related protein), *BSCL2* (Bernardinelli-Seip congenital lipodystrophy 2), *CXX1*, *DFNA5*, *hHLP1*, *NEU1* (sialidase 1) and *RBP4* were found to be methylated in cell lines but not in normal tissues. Representative sequencing results are shown in Supplementary Figure 2.

The methylation status of *CXX1*, *FLJ13072*, *HBA1*, *hHLP1* and *RBP4* inversely correlated with their expression status (Figure 2 and Table 1). For example, HT29 and DLD-1, but not HCT116, silenced the expression of *CXX1* and *HBA1*, and harbored CpG methylation of the gene promoter. The expression of *RBP4* was detected and methylation of the gene was not observed in HT29. *DFNA5* was methylated and silenced in all cell lines tested (HT29, DLD-1, RKO, SW48, Lovo and SW480; Figures 2 and 3c, and Supplementary Figure 2A) except for HCT116 that expressed low level of *DFNA5* at baseline. We could not find an inverse correlation between basal expression and promoter methylation in *ADFP*, BSCL and *NEU1*, suggesting alternate mechanisms of regulation.

Promoter hypermethylation in primary CRC

Five genes (*CXX1*, *DFNA5*, *HBA1*, *hHLP1* and *RBP4*) exhibited a profile of complete absence in two of three CRC cell lines and common reactivation by 5-aza-dC treatment. They also harbored methylation in cell lines but not in normal colon tissues. Thus, we investigated promoter methylation of these genes in 10 pairs of matched CRC and colon normal tissues by combined

Table 1 Methylation profiles of colorectal cancer (CRC) cell lines and normal colon tissues

			MSI+	*MSI−*	*MSI+*				
No.	*Accession no.*	*Gene name*	*HCT116*	*HT29*	*DLD1*	*Normal 1*	*Normal 2*	*Normal 3*	*Normal 4*
1	BC005127.1	ADFP	M	M	M	U	U	U	U
2	AL096741.1	ASC1p100	U	*	*	U	U	U	*
3	BC004911.1	BSCL2	*	M	*	U	U	U	U
4	U63131.1	CDC37	U	U	U	*	U	*	U
5	NM_019886.1	CHST7	U	*	U	*	*	U	*
6	NM_001834.1	CLTB	U	U	U	U	U	U	U
7	NM_003928.1	CXX1	U	M	M	U	U	U	U
8	NM_004403.1	DFNA5	M	M	M	U	U	U	U
9	AK023134.1	FLJ13072	M	M	M	M	M	M	M
10	AF349571.1	HBA1	U	M	M	U	M/U	M/U	U
11	U38371.1	hHLP1	M	M	M	U	U	U	U
12	AF173154.1	HYAL1	M	M	M	M	M	M	M
13	AH006662	LIS1	U	U	U	U	U	U	U
14	NM_015846.1	MBD1	U	U	U	*	U	*	U
15	U84246.1	NEU1	U	U	M	U	U	U	U
16	NM_013959.1	NRG1	U	U	U	U	U	U	U
17	NM_006744.2	RBP4	M	U	M	U	U	U	U
18	NM_006867.1	RBPMS	U	U	U	U	U	U	U
19	NM_003003.1	SEC14L1	U	U	U	U	U	U	U
20	NM_015515.1	TP53I3	*	U	U	*	*	U	*
21	M61715.1	WARS	U	U	U	U	U	U	U

Abbreviations: M, harbor methylated alleles; M/U, harbor both methylated and unmethylated alleles; U, harbor unmethylated alleles. Gene expression was absent in three CRC cell lines by microarray results. *, not assessed. Norma1 1–4, samples from patients No. 24, 33, 34 and 36, respectively.

bisulfite-restriction analysis (COBRA; Supplementary Figure 1). In three CRC cell lines and normal tissues, we performed COBRA in parallel to confirm the bisulfite-sequencing results of each gene. The results from the parallel analysis were also considered as positive and negative controls for primary tissues due to their perfect consistency with the sequencing results except for *CXX1* in normal tissues (Supplementary Figure 1A). The frequency of tumor methylation of *DFNA5* was (40%, 4/10), and interestingly, no methylation of *DFNA5* was found in all normal colon tissues tested (0%, 0/9), indicating that the gene harbored cancer-specific promoter methylation. Thus, we focused on *DFNA5* for further study.

DFNA5 is frequently methylated and downregulated in primary colon cancers

To study *DFNA5* promoter methylation in human primary tissues, quantitative-methylation specific PCR (TaqMan-MSP) analysis with a probe targeted to the CpG island of *DFNA5* was performed in 100 pairs of colon normal tissues (PN) and primary CRC (PT) as well as three CRC cell lines (HCT116, HT29 and DLD-1) that included samples previously analysed by bisulfite-sequencing and COBRA (Figure 3a). Eleven colon normal mucosa tissues of the noncancer patients (NN) were also included to compare methylation specificity between cancer and noncancer patients. In 100 pairs of colon samples, methylation values (TaqMan methylation values, TaqMeth V) in tumor ranged from 0 to 326.30 (median value 14.44), and in normal colon from 0 to 7.57 (median value 0.001). The overall TaqMeth V levels detected in primary CRC (24.12 ± 44.67, mean $\pm$ s.d., $n = 100$) were also significantly higher than that in corresponding normal tissues (0.15 ± 0.89, mean $\pm$ s.d., $n = 100$; $P < 0.001$, Student's *t*-test; Table 2). *DFNA5* was thus frequently methylated in primary CRC tissues but displayed absent or minimal levels of methylation in corresponding normal tissue. Representative sequencing results of DFNA5 in CRC cell lines and tissues are shown in Supplementary Figure 2A.

Examining TaqMeth value (V), we found that cut-off values from 0.1 to 10 were all statistically significant by χ^2 analysis (normal vs tumor and methylation vs cases without methylation. Methylation of DFNA5 showed highly discriminative receiver–operator characteristic (ROC) curve profile, clearly distinguishing CRC from corresponding normal mucosa ($P < 0.001$; Figure 3b). The optimal cut-off (value, 0.65) was calculated from the ROC curve in order to maximize sensitivity and specificity. At this cut-off, the sensitivity was 65% (65/100) and specificity was 97% (97/100; $P < 0.001$; Table 2). Only 1 (TaqMeth V = 2.18) out of 11 colon epithelial samples from noncancer patients and only 3 out of 100 of paired normal colon mucosa from cancer patients (patients' no. 39, 65, 75) displayed a TaqMeth V over 0.65 (Figure 3a). The methylation level in all remaining 10 cases of normal colon epithelium was completely undetectable, and 87 cases of matched normal colon mucosa harbored levels lower than 0.001. A high level of *DFNA5* promoter methylation was also found in CRC cell lines (3/3, 100%), consistent with the bisulfite sequencing and COBRA results. Taken together, DFNA5 was frequently methylated in primary CRC tissues but at minimal levels in corresponding normal tissue.

We next performed RT–PCR analysis using cDNA prepared from other colon cancer cell lines (RKO,

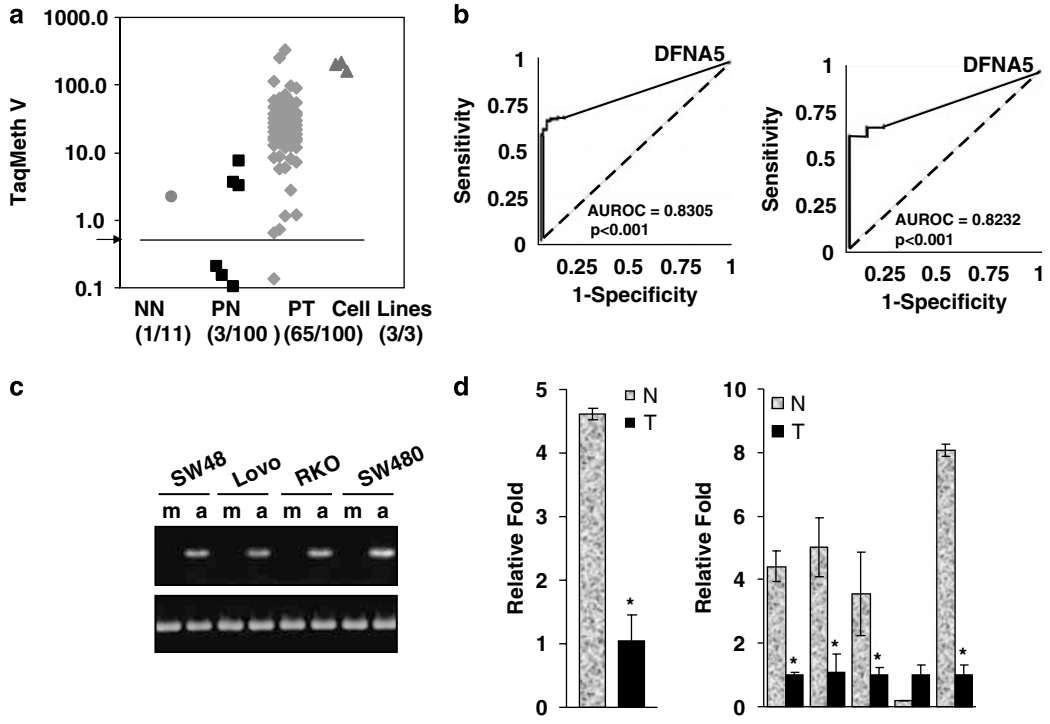

Figure 3 Quantitative level of methylation and downregulation of DFNA5 in primary colon cancer. (**a**) Scatter plot of *DFNA5* promoter methylation. Sixty-five of 100 colorectal cancer (CRC) primary tumors (PT) harbored values above the optimal cut-off value 0.65. Only 1 of 11 normal colon epithelial tissues (NN) and 3 of 100 paired normal colon tissue (PN) were above the cut-off ($P<0.001$, χ^2 test). CRC cell lines tested were HCT116, HT29 and DLD-1. Arrow indicates the cut-off value of 0.65. Samples with a ratio equal to zero could not be plotted correctly on a log scale, so are presented here as 0.001. All assays were performed in duplicate format, and experiments were repeated twice. Data showed reproducible and concordant results. TaqMeth V is described in Materials and methods. (**b**) ROC curve analysis of TaqMeth V of DFNA5. Left, the Area under ROC (AUROC, 0.8305) conveys the accuracy in distinguishing matched normal colon (PN) from CRC (PT) in terms of its sensitivity and specificity ($P<0.001$). The optimal cut-off was calculated from this ROC analysis. Right, ROC curve analysis of DFNA5 in PT vs normal colon tissue from patients without cancer (NN). AUROC was 0.8232 ($P<0.001$). Solid line, DFNA5; dashed line, no discrimination. (**c**) Reverse transcription (RT)–PCR analysis of *DFNA5* in colon cancer cell lines. Expression of *DFNA5* was robustly reactivated by 5-aza-dC. β-Actin expression is shown as a loading control. m, no treatment; a, 5-aza-dC treatment. (**d**) Real-time-PCR was performed in cDNA prepared from CRC patients or patients without cancer. *Left*, cDNA was prepared from a CRC patient (T) or a patient without cancer (N). Relative fold was calculated by expression of *DFNA5* mRNA relative to GAPDH (an internal control) as described in Materials and methods. *Right*, Expression of DFNA5 was examined in matched normal and tumor cDNAs from CRC patients by real-time RT–PCR. N, normal tissue cDNA; T, CRC tissue cDNA. $*P<0.005$.

SW48, SW480 and Lovo), and observed that *DFNA5* was silenced all these cell lines (Figure 3c). Methylation of DFNA5 in these cell lines was confirmed by bisulfite sequencing (Supplementary Figure 2A, and data not shown). *DFNA5* expression increased after 5-aza-dC treatment, indicating that the expression of *DFNA5* strictly correlated with the promoter methylation. To examine mRNA levels of *DFNA5* in human tissue, real-time PCR was performed in cDNA prepared from CRC patients or patients without cancer (Figure 3d). In comparison with normal colon tissue cDNA from a patient without cancer and tumor cDNA from a colon cancer patient, the *DFNA5* expression in tumor was five times lower than in normal tissue ($P=0.007$; Figure 3d, left). In five pairs of matched normal and tumor cDNAs, four cases showed downregulation of DFNA5 in tumor (Figure 3d, right).

DFNA5 has tumor suppressive activity in CRC cell lines

We then elucidated the function of *DFNA5* as a suppressor of cancer cell growth. We selected a stable clone (clone no. 6) from HCT116 expressing relatively high level of DFNA5 (Supplementary Figure 3A). First, we compared *in vitro* cell growth with clones over-expressing *DFNA5* or with vector control by the MTT assay. Cell growth in a clone overexpressing *DFNA5* decreased to 64% of control ($P<0.05$, Student's *t*-test) (Figure 4a). We also performed a colony focus assay after incubating cells in the presence of G418 selection for 2 weeks. In control cells (vector only), a number of colonies grew (168.00 ± 21.38 colonies) whereas in the cells with overexpression of the *DFNA5*, colony numbers were markedly decreased (6.00 ± 3.60) ($P<0.001$, Student's *t*-test; Figure 4b). Similar results were observed in transiently transfected cells (Supplementary Figure 3B). To determine the inhibitory ability of *DFNA5* in anchorage-independent cell growth, we assessed colony formation in soft agar. We found a marked decrease in the number of colonies in stable cells overexpressing *DFNA5* compared with the control ($P<0.001$; Figure 4c). When we tested another clone stably expressing *DFNA5*, the results were similar (data not shown). These results indicate that *DFNA5* suppresses growth in colon cancer cells.

Table 2 Sensitivity and specificity of DFNA5 promoter methylation in colon cancer

Cut-off value	*%*	*%*		*TaqMeth V*		
	Sensitivity	*Specificity*	P-*value**	*PT*	*PN*	P-*value***
0.1	68 (68/100)	94 (94/100)	<0.001			
0.65	65 (65/100)	97 (97/100)	<0.001	24.12 ± 44.67	0.15 ± 0.89	<0.001
10	56 (56/100)	100 (100/100)	<0.001			

Sensitivity, positive methylation/total tumor cases; Specificity, negative methylation/total normal cases. TaqMeth V is expressed as mean ± s.d. **P*-value was calculated from the χ^2 test. ***P*-value was derived from the Student's *t*-test.

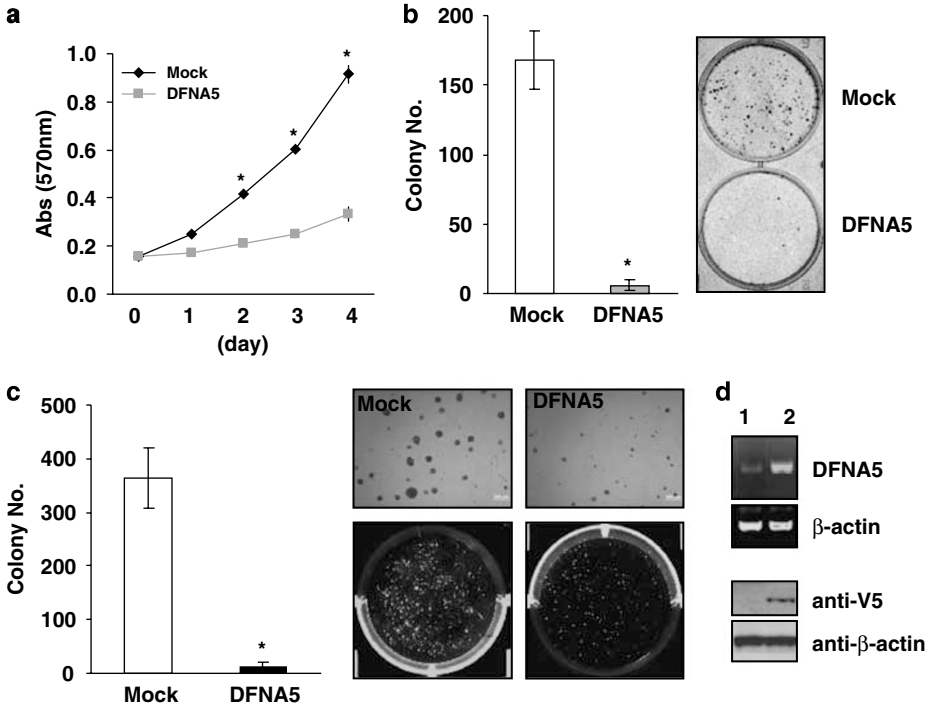

Figure 4 DFNA5 inhibits cell growth and colony forming ability. (**a**) MTT assay was performed in HCT116 stably overexpressing *DFNA5* or control cells. Cell growth was expressed as absorbance (Abs) at 570 nm wavelength. Experiments were done in triplicate, and values indicate means ± s.d. **P*<0.05. (**b**) Colony focus assays were performed in the stable cell lines. Colonies were counted and photographed under a microscope (picture in upper right) after 2 weeks of cell incubation in the presence of G418. (**c**) Inhibitory ability of *DFNA5* in anchorage-independent cell growth was determined by the soft agar assay. Right, colonies at size >0.5 mm were counted. Scale bar, 500 μm. Left, colonies were photographed under phase-contrast microscope (top) or under UV after staining with ethidium bromide in 1 × PBS/0.1% Triton X-100 solution overnight (bottom). Values are expressed as means ± s.d. and are derived from two independent experiments done in triplicate. **P*<0.05. (**d**) *DFNA5* expression was examined in stable cell lines by reverse transcription (RT)–PCR and western blotting with anti-V5 antibody. An antibody against endogenous *DFNA5* was not available. 1, mock; 2, *DFNA5* stable cells.

Next, we transiently transfected two individual siRNAs targeting *DFNA5* and a nontargeting control siRNA into HEK293, and performed the cell growth assay. We observed a significant increase of cell growth in HEK293 cells transfected with either siRNA-nos.1 or -2 (Figure 5a). We then transfected these siRNAs into HCT116 cells separately and incubated them for 4 days to determine cell growth and for 8 days to determine colony-forming ability. We found that knockdown of *DFNA5* substantially increased HCT116 cell growth (Figure 5b). Also, colony numbers were markedly increased in these cells (Figure 5c). Gene knockdown was confirmed by RT–PCR in each cell line. We also trypsinized HCT116 cells and re-seeded the same number of cells in culture dishes after transfection and subsequent incubation of cells, as previously described (Rochester *et al*., 2005). Similar results were observed in cell growth and colony numbers (data now shown).

In order to understand the mechanism of DFNA5 for rapid increase of cell proliferation, we examined factors involved in cell cycle transition after siRNA transfection into HCT116 cells. We found that expression of proteins involved in the G_1 to S transition including cyclins D1, D3 and E, as well as cyclin-dependent kinase (CDK) 4/6, but not CDK inhibitor p27 (Kip1), were increased in the cells transfected with siRNA-*DFNA5*. We also found that knockdown of *DFNA5* in HCT116 cells induced phosphorylation of Akt, one of the upstream

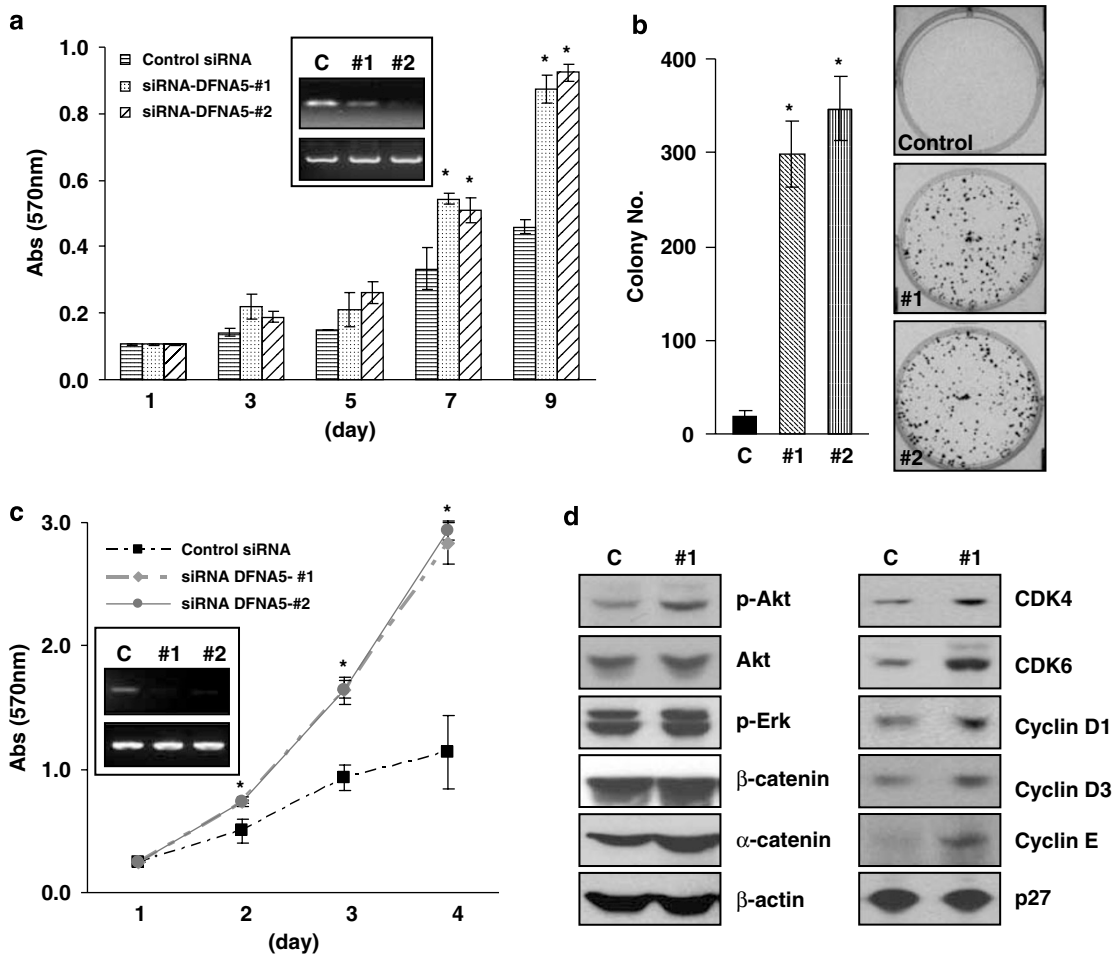

Figure 5 DFNA5 knockdown increases cell growth. MTT assay was performed in HEK293 (**a**) or HCT116 (**b**) after transfection of siRNAs targeting *DFNA5*. Experiments were done in triplicate, and values indicate means ± s.d. $*P<0.05$. *DFNA5* gene knock down was examined by RT–PCR in each cell lines. HEK293 expressed *DFNA5* (box, upper), but the gene methylation was not found (data not shown). β-Actin expression is shown as a loading control (box, lower). (**c**) Control siRNA; no. 1, siRNA-*DFNA5*-no. 1; no. 2, siRNA-*DFNA5*-no. 2. (**c**) Colony formation assay for HCT116 cells transiently transfected with siRNA-DFNA5 or siRNA-control was performed after 8 days of cell incubation. Colonies were counted (upper) and photographed under a microscope (lower). Colonies from nontransfectant HCT116 cells were not observed within 10 days of incubation after they were grown at the density of $1–2 \times 10^4$ per well of 6-well pates. Values are expressed as means ± s.d. and are derived from two independent experiments done in triplicate. $*P<0.05$. (**d**) Western blotting of whole cell lysates extracted from HCT116 transfected with *DFNA5* siRNAs. Since the biological effects of the two siRNAs in this study were similar, only siRNA-*DFNA5*-no. 1 was transfected into cells for western blotting. β-Actin was used as a loading control. C, control siRNA; no. 1, siRNA-*DFNA5*-no. 1.

proteins of the cyclins, but the phosphorylation of Erk remained unchanged (Figure 5d). These results imply that loss of *DFNA5* expression leads to an increase in cell cycle progression and cell growth potentially driven through the AKT pathway.

Discussion

CRC is the second leading cause of cancer and accounts for 10% of all new cancer cases and cancer deaths (Jemal *et al*., 2005). Mortality rates for colon cancer have fallen during the past 20 years because of early detection from increased screening. Finding potential diagnostic markers and therapeutic targets for CRC will aid early detection and treatment of CRC. Epigenetic events play key roles in the development and progression of CRC, and aberrant CpG island methylation of gene promoters is a common alteration in human CRC (Toyota *et al*., 2000). Genes with frequent promoter hypermethylation in TSGs shed light on the underlying mechanisms of carcinogenesis and may have prognostic or predictive value for CRC (Esteller *et al*., 2001; Lind *et al*., 2004; Xu *et al*., 2004). Based on pharmacological unmasking and subsequent applications of new algorithms, we identified candidate-methylated genes in human ESCC (Yamashita *et al*., 2002; Kim *et al*., 2006). In this study, the pharmacological unmasking of epigenetically silenced genes in CRC also uncovered new genes that were transcriptionally repressed. Methylation of some of the genes or gene families uncovered in our study has been described, such as CRBP1 (the RBP

subtype) in colon and prostate cancer (Goelz *et al*., 1985; Esteller *et al*., 2002; Jeronimo *et al*., 2004; Luo *et al*., 2005; Shutoh *et al*., 2005). *CXX1* is a CAAX box 1 gene localized at Xq26. Recently, the expression of *CXX1* was reported to be downregulated in human gastric cancer (Kim *et al*., 2005), and the gene promoter was methylated in human colon cancer (Suzuki *et al*., 2002; Lind *et al*., 2004; Xu *et al*., 2004). In our study, *CXX1* gene expression was tightly correlated with the promoter methylation, but the methylation frequency in normal colon tissues was also relatively high.

The *DFNA5* promoter was more interesting since it was frequently methylated in primary tissues (65%) compared to corresponding colon normal tissues (3%; cut-off value, 0.65), and the level of methylation was much higher in neoplastic than in normal tissues. The frequency of DFNA5 methylation in CRC ranks with other genes methylated at high frequency in CRC (cyclin A1, CDX1, RAR-β, MYOD1, p15 (INK4b) and COX-2; Xu *et al*., 2004). Primary tumor tissues were clearly discriminated from normal tissues by TaqMan-MSP analysis of the gene, indicating that detection of *DFNA5* promoter methylation in blood or stool DNA may have potential for identifying individuals with cancer.

Aberrant methylation of *DFNA5* was recently detected in 52% of primary gastric cancers (Akino *et al*., 2007), and we also found that 54% gastric cancer tissues (17/31 cases) and 0.9% matched normal tissues (1/11) displayed TaqMeth V over the optimal cut-off calculated in gastric cancer tissue (value 1, data not shown), supporting the quantitative analysis in this study as a reliable method for detection of *DFNA5* methylation. The DFNA5 methylation was also examined in human esophageal and bladder tumor tissue by the TaqMan-MSP analysis. The frequency of the DFNA5 methylation in both types of cancer was relatively low (less than 20%), and no detectable level of methylation was observed in corresponding normal tissues (data not shown).

The mRNA level of *DFNA5* in colon cancer was much lower than in normal tissues by real-time PCR, and all CRC cell lines tested that were completely methylated in the *DFNA5* promoter were silenced or weakly expressed. A report of a close association of *DFNA5* gene silencing with methylation of a 'D' region located near Exon 1 (Akino *et al*., 2007) indicates that methylation of specific CpG sites in the *DFNA5* gene may be necessary to shut down its expression completely. Thus, the discrepancy between expression and promoter methylation of *DFNA5* in HCT116 can be explained by no methylation found in the 'D' region in HCT116 cells (data not shown).

Transcription of *DFNA5* decreases in etoposide-resistant melanoma cells, and enhanced expression of the gene increases the cellular susceptibility to etoposide-induced apoptosis (Lage *et al*., 2001). CRC is generally not sensitive to etoposide in the clinical setting. In addition, p53 regulation of DFNA5, a role for *DFNA5* in p53-mediated cellular response to DNA damage and a pro-apoptotic effect of *DFNA5* (Masuda *et al*., 2006; Akino *et al*., 2007) were recently reported. In our study, we also observed little inhibitory effect of *DFNA5* on the growth of DLD-1 (p53-mt) and HCT116-p53-null cells (data not shown). These results imply that *DFNA5* might be involved in a cellular pathway leading to p53-dependent apoptosis.

Identification of cancer-specific methylation with high frequency and silencing of *DFNA5* in CRC implicates this gene as a common inactivation event in colon tumorigenesis. Detection and quantification of *DFNA5* methylation deserves further attention as a diagnostic biomarker due to its high frequency in primary tumors and near absence in normal tissues. Detection of *DFNA5* methylation levels in stool and/or plasma DNA may prove valuable in the diagnosis and monitoring of patients with colon cancer. DFNA5 also has a suppressive effect on the growth of colon cancer cells. Further evaluation of its effect on the AKT pathway and other mechanisms of growth regulation may provide new therapeutic approaches for the treatment of colon cancer patients.

Materials and methods

Cell lines and tissues

CRC cell lines, HCT116, HT29, DLD-1, RKO, SW48, SW480 and Lovo were purchased from ATCC (Manassas, VA, USA). CRC cell lines were grown in McCoy, 5 × supplemented with 10% fetal bovine serum (FBS). HEK293 were obtained from ATCC and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% EBS. One hundred primary CRC and matched colon normal adjacent mucosa tissue were obtained from patients who underwent surgery at the Medical Institute of Bioregulation Hospital, Kyushu University. Eleven normal colon epithelial samples were obtained from patients with cancer at Department of Pathology, The Johns Hopkins University.

Microarray and RT–PCR analysis

We performed oligonucleotide microarray analysis on the GeneChip Human Genome U133A Array (Affymetrix) containing 22 284 genes, as per the manufacturer's instruction. Data from the array is analysed using GeneChip DNA Analysis Software (Affymetrix) to obtain genotype and signal information. We identified genes absent at baseline or upregulated by pharmacological treatment according to the manufacturer's algorithm. For RT–PCR, cells were treated with 5 μM 5-aza-dC (Sigma, St Louis, MO, USA) every 24 h for 3 days. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed with Superscript II reverse transcriptase (Invitrogen). RT–PCR was performed at 30 cycles: 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min. PCR products were gel extracted and sequenced to verify true expression of the genes. The primer sequences for RT–PCR are shown in Supplementary Table 2.

Bisulfite sequencing

Bisulfite-modified genomic DNA was amplified by PCR using 10 × buffer (166 mM $(NH_4)_2SO_4$, 670 mM Tris Buffer (pH 8.8), 67 mM $MgCl_2$, 0.7% 2-mercaptoethanol, 1% DMSO) and primer sets that were designed to recognize DNA bases after bisulfite treatment. The conditions for PCR amplifications were as follows: a 5 min of incubation at 95 °C was followed by 45 cycles of 1 min at 95 °C, 1 minute at 54 °C and 2 min at

72 °C. A 7-min elongation step at 72 °C completed the PCR amplification. PCR products were gel extracted (Qiagen, Valencia, CA, USA) and sequenced with an internal primer (F2) using the ABI BigDye cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). When methylation is found in more than 20% of total CGs in amplified PCR products, it is considered as 'methylation positive'. When methylated and unmethylated alleles are observed in chromograms, it was considered as 'partial methylation (M/U)'. Search for CpG islands in each gene promoter was done by using the online accessible software (Methprimer). Bisulfite-sequencing primers were designed at the CpG islands within 1 or 2 kb upstream of the transcription start site (TSS), and primer sets were shown in Supplementary Table 3.

Combined bisulfite-restriction analysis
PCR-amplified products were run on a 1.5% agarose gel stained with ethidium bromide. The PCR bands were excised and purified using a gel extraction kit (Qiagen), and resuspended in 30 μl of water. DNA (15 μl) was digested with *BstU*1 (New England Biolabs Inc., Beverly, MA, USA) at 60 °C overnight. Samples were loaded on a 10% acrylamide gel, stained with 1 × SYBR Green Gold (Molecular Probes, Eugene, OR, USA) and visualized under UV light.

Quantitative-methylation specific PCR
For quantitative methylation analysis, PCR primers were designed to hybridize to the region of *DFNA5* that was determined to be methylated in CRC cell lines by bisulfite sequencing, and a fluorescent probe was synthesized to the amplified region of the DNA. All oligonucleotide primer pairs were purchased from Invitrogen, and the TaqMan probe from VWR (West Chester, PA, USA). The *DFNA5* primers had the following sequences: 5′-CTAATTAAACCGTAAAACC GCG-3′ (*DFNA5* TAQ F) and 5′-TTTTTCGTTGCGC GGGATCG-3′ (*DFNA5* TAQ R). The *DFNA5* probe was 6FAM 5′-ATTCGACCCCGCGAAAAAACGCCGCT-3′-TAMRA. All protocols for TaqMan-MSP were performed as reported (Kim *et al*., 2006), and all reactions were performed in duplicate. To ensure the specificity of the TaqMan-MSP analysis, each 384-well PCR plate had wells that contained bisulfite-converted DNA isolated from patient tissue samples and wells that contained the following controls: *in vitro* methylated lymphocyte DNA (positive control), DNA from normal colon mucosa in which *DFNA5* is not methylated (negative control) and multiple water blanks (control for PCR specificity). Lymphocyte DNA from a healthy individual was methylated *in vitro* with excess *Sss*I methyltransferase (New England Biolabs Inc.) to generate completely methylated DNA, and serial dilutions (90–0.009 ng) of this DNA were used to construct a calibration curve for each plate. All samples were within the assay's range of sensitivity and reproducibility based on amplification of internal reference standard (threshold cycle (CT) value for β-actin of $\leqslant 40$). The methylation ratio was defined as the quantity of fluorescence intensity derived from the *DFNA5* promoter amplification divided by fluorescence intensity from β-actin amplification, and multiplied by 100 (TaqMan methylation value: TaqMeth V). This ratio was used as a measure for the relative level of methylated *DFNA5* DNA in samples. The samples were categorized as unmethylated or methylated based on the sensitivity of the assay.

Statistical analysis
We used the methylation levels (TaqMeth V) for DFNA5 to construct receiver operating characteristic (ROC) curves for the detection of CRC. Using this approach, the AUROC (area under ROC) identified optimal sensitivity and specificity levels (that is, cut-offs) at which to distinguish normal from malignant CRC tissues, and corresponding TaqMeth V threshold was calculated. The cut-off values determined from ROC curve were applied to determine the frequency of DFNA5 methylation. Samples with TaqMeth V, 0.65 or higher were designated as methylated, and samples containing less than TaqMeth V of 0.65 were designated as unmethylated. Statistical analyses were conducted using STATA Version 9 (STATA Inc., College Station, TX, USA).

Real-time RT–PCR
cDNA panel of human normal colon tissue and colon cancer tissue were purchased from BioChain Institute Inc. (Hayward, CA, USA), and five matched normal and tumor cDNA were purchased from Clontech Laboratories Inc. (Mountain View, CA, USA). One micro liter each of 5 × diluted cDNA was used for the assay using QuantiFast SYBR Green PCR Kit (Promega, Valencia, CA, USA). Primer sequences were 5′-ACCAATTTCCGAGTCCAGTG-3′ (*DFNA5* cDNA Real F), 5′-CAGGTTCAGCTTGACCTTCC-3′ (*DFNA5* cDNA Real R), 5′-CAACTACATGGTTTACATG-3′ (GAPDH F) and 5′-GCCAGTGGACTCCACGAC-3′ (GAPDH R). Amplifications were carried out in 384-well plates in a 7900 Sequence Detector System (Perkin-Elmer Applied Biosystems, Norwalk, CT, USA). Thermal cycling was initiated with a first denaturation step at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s. Expression of *DFNA5* mRNA relative to GAPDH was calculated based on the threshold cycle (C_t) as $2^{-\Delta(\Delta Ct)}$, where $\Delta C_t = C_{t,DFNA5} - C_{t,GAPDH}$ and $\Delta(\Delta C_t) = \Delta C_{t,N} - \Delta C_{t,T}$ (N, normal tissue cDNA; T, tumor tissue cDNA).

Plasmid construction and stable cell lines
pAd-*DFNA5* plasmid was kindly provided by Dr Hermann Lage (Humboldt University, Germany) and used for a template for cDNA synthesis of *DFNA5*. Full-length *DFNA5* was synthesized by PCR using Platinum Pfx DNA polymerase (Invitrogen), and cloned into pcDNA3.1 expression vector using the pcDNA3.1 Directional TOPO Expression Kit (pcDNA3.1/V5-His Topo vector) as manufacturer's protocol (Invitrogen). Plasmid sequence analysis was performed to confirm the fidelity of the DFNA5 insert (data not shown). HCT116 cells were transfected using Fugene-6 (Roche, Basel, Switzerland) in OPTI-MEM (Invitrogen) as per the manufacturer's instructions. Colonies were selected for stable cell lines after transfected cells were grown in the presence of G418 (1 mg ml^{-1}) for 2 weeks. G418-resistant control clones were pooled as a control cell line. The expression of *DFNA5* in the cell line was confirmed by RT–PCR and by western blotting with anti-V5 antibody after extraction of whole cell lysates.

Cell growth assay
HCT116-*DFNA5* stable cells were plated in a 12-well plate at a density of 2–3 × 10^4 cells per well and incubated at 37 °C. The tetrazolium-based cell viability (MTT) assay was performed every day for 4 days. The results were expressed as an absorbance at 570 nm wavelength.

Colony focus assay
HCT116-*DFNA5* stable cells were plated in a 6-well plate at a density of 200 cells per well and incubated in the presence of G418 (1 mg ml^{-1}) for 2 weeks and stained with 0.4% crystal violet solution (MeOH/acetic acid, 3:1). After air drying,

colonies were photographed under the microscope and counted. Two independent experiments were performed and each experiment was done in triplicate.

Soft agar assay
HCT116-*DFNA5* cells (1×10^4) were seeded in 1 ml of 0.3% low-melting agarose over a 0.6% agar bottom layer in McCoy, 5 × supplemented with 10% FBS and 1 mg ml^{-1} of G418. The medium was changed three times a week and the clones were allowed to grow for 10 days. Two independent experiments were performed and each experiment was done in triplicate.

Knockdown of DFNA5 *and cell growth assay*
Two individual siRNAs targeting *DFNA5* gene and nontargeting control siRNA were purchased from Dharmacon (Chicago, IL, USA). On-target plus set of 2 duplex (05 and 06) was re-labeled as siRNA-*DFNA5*-nos.1, and -2, respectively. A 50 nM portion of each siRNA was transiently transfected to HEK293 or HCT116 using LipofectamineRNAiMax transfection reagent (Invitrogen) in OPTI-MEM. After 24 h, cells were incubated in complete growth medium. Initial cell seeding density was 3×10^3 per well in 96-well plates for HEK293 and 2×10^4 per well in 12-well plates for HCT116, and MTT assay was performed at indicated time points. For colony focus assay, HCT116 (1×10^4 per well of 6-well plates) were transfected with siRNA and incubated for 8 days after addition of growth medium.

Western blot analyses
Whole cell lysates were separated on 4–12% gradient SDS–PAGE and transferred to nitrocellulose. The blots were incubated with anti-V5 (Invitrogen), anti-AKT, anti-phospho-AKT (Ser473), anti-phospho-Erk (Thr202/Tyr204), anti-cyclin D1, anti-cyclin D3, anti-cyclin E, anti-CDK4, anti-CDK6, anti-p27, anti-PCNA, anti-α-catenin, anti-β-catenin or anti-β-actin antibody (Cell Signaling Technologies, Danvers, MA, USA) for 2 h at room temperature or 4 °C overnight. After antibody washing, the blots were reacted with their respective secondary antibody and detected with enhanced chemiluminescence reagents (Amersham, Pittsburgh, PA, USA) according to the supplier's protocol. All antibodies were purchased from Cell Signaling Technology except anti-V5 and anti-β-actin (Sigma) antibodies.

Acknowledgements

Under a licensing agreement between OncoMethylome Sciences, SA and the Johns Hopkins University, Dr Sidransky is entitled to a share of royalty received by the university on sales of products described in this article. Dr Sidransky owns OncoMethylome Sciences, SA stock, which is subject to certain restrictions under the university policy. Dr Sidransky is a paid consultant to OncoMethylome Sciences, SA and is a paid member of the company's Scientific Advisory Board. The term of this arrangement is being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).