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Epigenetic regulation of the Wnt signaling inhibitor *DACT2* in human hepatocellular carcinoma

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Abbreviations: HCC, hepatocellular carcinoma; *DACT2*, Dapper, a Dishevelled-associated antagonist of β-catenin, homolog 2; MSP, methylation specific polymerase chain reaction; BSSQ, Bisulfite sequencing; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TSS, transcription start site; M, methylated; U, unmethylated; 5-AZA, 5-aza-2'-deoxycytidine; NL, normal blood lymphocyte DNA; IVD, in vitro methylated DNA; PDZ, Post synaptic density-95/Discs large/Zonula occludens-1; PBS, phosphate-buffered saline; AJCC, American Joint Committee on Cancer; AFP, alpha-fetoprotein; ALTSG, American Liver Tumor Study Group; BrdU, bromodeoxyuridine; TCF, T-cell factor; LEF, lymphoid enhancer factor; NT, non-tumor tissue; T, tumor tissue; SNP, single-nucleotide polymorphism

DACT2 (Dapper, Dishevelled-associated antagonist of β-catenin homolog 2) is a member of the DACT family involved in the regulation of embryonic development. Human *DACT2* is localized on 6q27, a region of frequent loss of heterozygosity in human cancers. However, the regulation of *DACT2* expression and function in hepatocellular carcinoma (HCC) remains unclear. In this study, genetic and epigenetic changes of *DACT2* were analyzed in HCC cell lines and primary cancer. We found no single-nucleotide polymorphism (SNP) associated with HCC. Promoter region methylation was correlated with loss or reduction of *DACT2* expression, and restoration of *DACT2* expression was induced by 5-aza-2'-deoxycytidine (5-AZA) in HCC cell lines. Promoter region methylation was found in 54.84% of primary HCC. Reduction of *DACT2* expression was associated with promoter hypermethylation, and expression of *DACT2* was inversely related to β-catenin expression in primary HCC. *DACT2* suppressed cell proliferation, induced G_2-M arrest in cell lines and inhibited tumor growth in xenograft nude mice. The transcriptional activity of *TCF-4* and the expression of Wnt signaling downstream genes were suppressed by *DACT2* re-expression and reactivated by depletion of *DACT2*. In conclusion, *DACT2* is frequently methylated in HCC and its expression is regulated by promoter hypermethylation. *DACT2* suppresses HCC by inhibiting Wnt signaling in human HCC.

Introduction

In men, hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer worldwide and the second cause of global cancer death. In women, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer death.[1] Carcinogenesis is a multi-step process with cumulative genetic and epigenetic changes involving oncogenes and tumor suppressor genes.[2-6] An increasing number of publications are focusing on identifying novel genes regulated by DNA methylation, histone modification and miRNAs.[7-12] These studies are mainly focused on elucidating the mechanisms of inactivation of tumor suppressors in different signaling pathways in order to find new therapeutic strategies in human cancer, including HCC. Wnt signaling plays an important role in the physiology, development, cell differentiation, proliferation and growth.[13] Abnormal activation of Wnt signaling is a major driving force in HCC.[14-16]

Dapper, a Dishevelled-associated antagonist of β-catenin (DACT), was isolated by a screen for proteins interacting with Dishevelled, a key factor in the Wnt signaling. Dapper and Dishevelled were co-localized intracellularly and formed a complex with Axin, GSK3 and β-catenin.[17] Human *DACT1* and *DACT2* were identified by Katoh et al. in 2003.[18] Human and murine *DACT3* were both identified by Fisher et al.[19] *DACT1* has been reported frequently to be methylated in HCC, and

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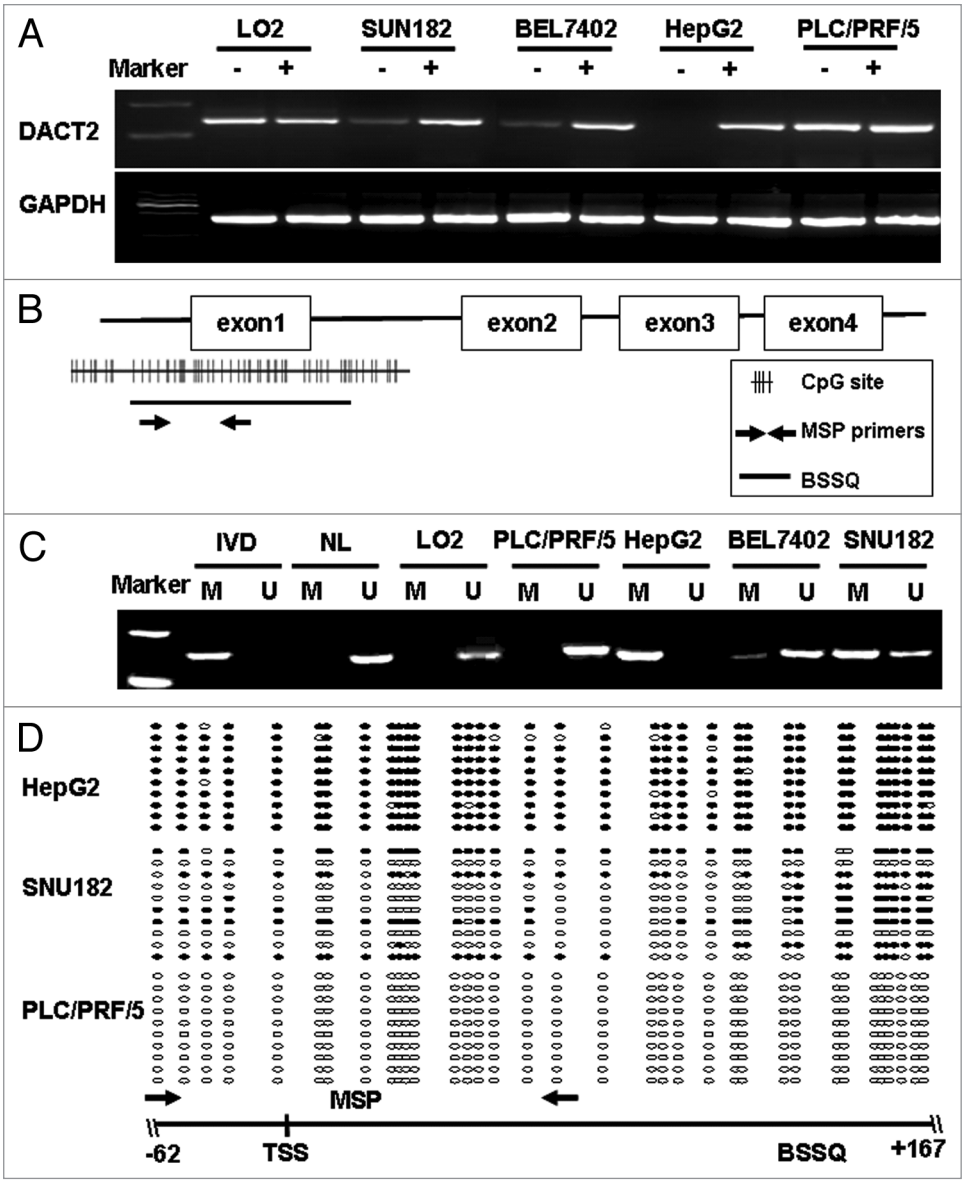

Figure 1. *DACT2* is silenced by promoter region hypermethylation in HCC cell lines. (**A**) Expression of *DACT2* was analyzed by semiquantitative RT-PCR in HCC cell lines and one immortalized hepatocyte cell line (LO2). (-) 5-AZA untreated; (+) 5-AZA treated; GAPDH was used as an internal control for RT-PCR. (**B**) Distribution of CpG sites in the promoter region of *DACT2* and the location of the MSP primers as well as BSSQ region are shown. (**C**) *DACT2* methylation was determined by MSP in HCC cell lines and LO2. IVD, in vitro methylated DNA (methylation control); NL, normal blood lymphocyte DNA (unmethylated control); M, methylated band; U, unmethylated band. (**D**) Promoter region methylation status of *DACT2* was analyzed by BSSQ in three HCC cell lines. Open circles denote unmethylated CpG site and filled circles represented methylated CpG site. The region amplified by MSP is indicated by arrows. TSS, transcription start site.

DACT3 has been found to be regulated by histone modifications in colorectal cancer.[12,20] Human *DACT2* is localized in chromosome 6q27, a region of frequent loss of heterozygosity in human cancers.[18,21-27] However, the regulation of *DACT2* expression and its function in human HCC remains unknown.

In this study, we first analyzed genetic and epigenetic changes of *DACT2*, and then studied its expression and function in hepatic carcinogenesis in vitro and in vivo.

Results

No single-nucleotide polymorphism (SNP) in the *DACT2* gene is associated with HCC. The sequencing of the full length cDNA and genomic DNA of *DACT2* in seven hepatic cancer cell lines and one immortalized hepatocyte cell line (LO2) revealed five SNP in exon 4, an important functional region also known as PDZ (post synaptic density-95/discs large/zonula occludens-1) binding domain.[28] Although no new mutations were discovered, four of the above SNPs were found both in patients with HCCs and in healthy controls. The respective locations and frequencies of these SNPs in both patients with HCCs and in healthy controls are as follows: 26.25% vs. 23.10% for A/G (rs6925614), 2.50% vs. 1.28% for T/C (rs79931308), 15.00% vs. 15.38% for A/C (rs10945501) and 1.25% vs. 1.28% for G/T (rs73789362). No significant differences were found in SNPs between HCCs patients and healthy individuals ($p > 0.05$).

***DACT2* is silenced by promoter hypermethylation in HCC cell lines.** *DACT2* was silenced in the HepG2 cell line and reduced in cell lines SNU182, BEL7402, SMMC7721 and SNU449. *DACT2* was normally expressed in PLC/PRF/5, 97H and in the immortalized cell line (LO2) (**Fig. 1A**). To investigate if silencing of *DACT2* is associated with promoter region hypermethylation, we first analyzed the CpG island of *DACT2* DNA sequence using a CpG Island search program (http://cpgislands.usc.edu). One CpG island was found in the promoter region (**Fig. 1B**). Then *DACT2* promoter region methylation was analyzed by MSP and bisulfite sequencing (BSSQ). Complete methylation was found in the HepG2 cells, and partial methylation was observed in the SNU182, BEL7402, SMMC7721 and SNU449 cell lines. No methylation was detected in LO2, PLC/PRF/5 and 97H cell lines (**Fig. 1C**). The methylation density within *DACT2* promoter region was characterized and validated by BSSQ (**Fig. 1D**). BSSQ of 10 individual clones of PCR products from HepG2 revealed dense methylation of CpGs within the promoter

region. The mixed methylation pattern of CpGs observed with BSSQ in the SNU182 cell line may represent both methylated and unmethylated alleles or both methylated and unmethylated clonal subpopulations within cultured cells. No methylation was found by BSSQ in PLC/PRF/5 and LO2. These results indicate that our MSP assays results accurately represent *DACT2* promoter region methylation status in these cell lines.

Concomitant loss of *DACT2* expression together with promoter region complete methylation was found in HepG2 cells. Normal expression without concomitant methylation was observed in LO2, PLC/PRF/5 and 97H cells. Partial methylation and reduced expression were detected in SNU182, BEL7402, SMMC7721 and SNU449 cell lines. These results indicate that promoter region methylation is correlated with *DACT2* silencing. *DACT2* expression was restored after 5-AZA treatment in HepG2 cells, and increased expression was observed in the SNU182 and BEL7402 cell lines. All of the above results demonstrated that *DACT2* expression was regulated by promoter region hypermethylation.

***DACT2* is frequently methylated in primary HCCs.** *DACT2* promoter region hypermethylation was not limited to cultured HCC cell lines. Frequent methylation was found in primary HCC (**Fig. 2A**). In 62 HCCs, 34 cases (54.84%) were methylated and 28 cases (45.16%) were unmethylated. No association was found between *DACT2* methylation and clinicopathological variables such as age, gender, hepatitis B/C virus infection, cirrhosis, AFP levels, tumor size or tumor stage in HCCs (**Table 1**).

***DACT2* expression is associated with promoter hypermethylation in primary HCCs.** *DACT2* expression was analyzed by IHC in tumor tissues and adjacent non-tumor tissues. *DACT2* was expressed in the cytoplasm of hepatic cells and its expression was reduced significantly in tumor tissues compared with adjacent tissues ($p < 0.01$) (**Fig. 2B and C**). Reduced *DACT2* expression was related to younger age ($p < 0.01$) and high serum levels of AFP ($p < 0.05$) patients (**Table 1**). These results suggest that *DACT2* may be a potential prognosis marker.

The association of *DACT2* expression and methylation status was analyzed in 62 HCC patients. Reduced expression of *DACT2* was found in 42 cases of tumor tissue, of which 28 cases were methylated (66.67%). Twenty cases were normally expressed, of which 6 cases were methylated (30%). Reduced *DACT2* expression was significantly related to promoter methylation ($p < 0.05$, **Figure 2D**). This suggests that *DACT2* expression may be regulated by promoter region methylation in primary HCC.

To evaluate whether activation of Wnt signaling was related to *DACT2* expression, β-catenin expression was analyzed by IHC in *DACT2* reduction cases. In cancer tissues, positive cytoplasm and nucleus β-catenin staining was found in 25 cases (59.52%). In adjacent tissues, β-catenin expression was mainly located on the membrane (31/42, 73%) (**Fig. 2E**). The expression of *DACT2* was associated with β-catenin location in HCC ($p < 0.05$).

HCC cell growth is suppressed by *DACT2* re-expression. Frequent loss of *DACT2* expression in HCCs suggests that it may be a potential tumor suppressor. To evaluate the effect of *DACT2* on cancer cell proliferation, colony formation assay was employed. *DACT2* expression resulted in significant suppression of long-term cell growth in colony formation assays, as much as 60% (**Fig. 3A**). Concomitantly, the inhibitory effect of *DACT2* was evaluated by BrdU incorporation assay. $HepG_2$ growth was inhibited about 30% by *DACT2* (**Fig. 3B**).

G_2-M arrest is induced by re-expression of *DACT2*. The effect of *DACT2* on the cell cycle was evaluated by flow cytometry. The ratio of G_2 phase was increased after re-expression of *DACT2* in HepG2 cells (G_1: 56.60 ± 1.72 vs. 51.70 ± 4.27; S: 25.95 ± 1.07 vs. 16.64 ± 2.50; G2:17.45 ± 2.81 vs. 31.0 ± 1.27) ($p < 0.01$) (**Fig. 3C and D**). These results indicate that *DACT2* induces G_2-M phase arrest in HCC.

***DACT2* has no effects on cell apoptosis.** To investigate the effects of *DACT2* on apoptosis, an annexin V-FITC assay was employed in HepG2 cells. No difference was found between the *DACT2* expression group and control group (11.98% ± 2.19 vs. 13.60% ± 1.67, $p > 0.05$) (**Fig. 3E and F**). These data suggest that *DACT2* is not involved in apoptosis in HCC cells.

Tumor growth is retarded by *DACT2* in vivo. Mice burdened with subcutaneous tumors were shown in **Figure 4A**. **Figure 4B** shows that the volume is significantly different in *DACT2* expressed and unexpressed HepG2 groups (251.09 ± 174.24 mm^3 vs. 533.20 ± 370.86 mm^3, $p < 0.05$). As shown in **Figure 4C and D**, the tumor weight is reduced in *DACT2*-expressing nude mice compared with an empty vector group (0.18 ± 0.11 g vs. 0.42 ± 0.25 g; $p < 0.05$). The results indicate that *DACT2* inhibits tumor growth in vivo.

***DACT2* is an inhibitor of Wnt signaling.** *DACT2* was reported to be involved in Wnt signaling during zebra fish and mice development. To explore the effects of *DACT2* on Wnt signaling, promoter-luciferase activity assays were employed in this study. The transcriptional activity of *TCF-4* was inhibited in both wild type and mutant β-catenin vectors groups by the re-expression of *DACT2* (**Fig. 5A**). Western blotting shows that the expression of TCF-4/β-catenin downstream targets, c-Myc and cyclin D1, was decreased after re-expression of *DACT2* in HepG2 cells (**Fig. 5B**). These results strongly suggest that *DACT2* is a Wnt signaling inhibitor in HCC.

To further analyze the effect of *DACT2* on Wnt signaling, *DACT2* was knocked down by RNA interference (RNAi) in PLC/PRF/5 cells. As shown in **Figure 5C and D**, the transcriptional activity of *TCF-4* was increased in *DACT2*-depleted cells, compared with control group, and the expression of Wnt signaling downstream genes was increased when depletion of *DACT2* in PLC/PRF/5 cells. These results suggest that *DACT2* is a Wnt signaling inhibitor in HCC.

Discussion

Abnormal activation of Wnt signaling is a major driving force in cancer, which can be initiated by genetic or epigenetic changes.[4,29] Frequent methylation of Wnt signaling antagonists suggest an important role for the activation of this pathway during carcinogenesis.[5,30]

DACT2 is located on human chromosome 6q27, a region frequently associated with loss of heterozygosity in human cancers.[18,21-27]

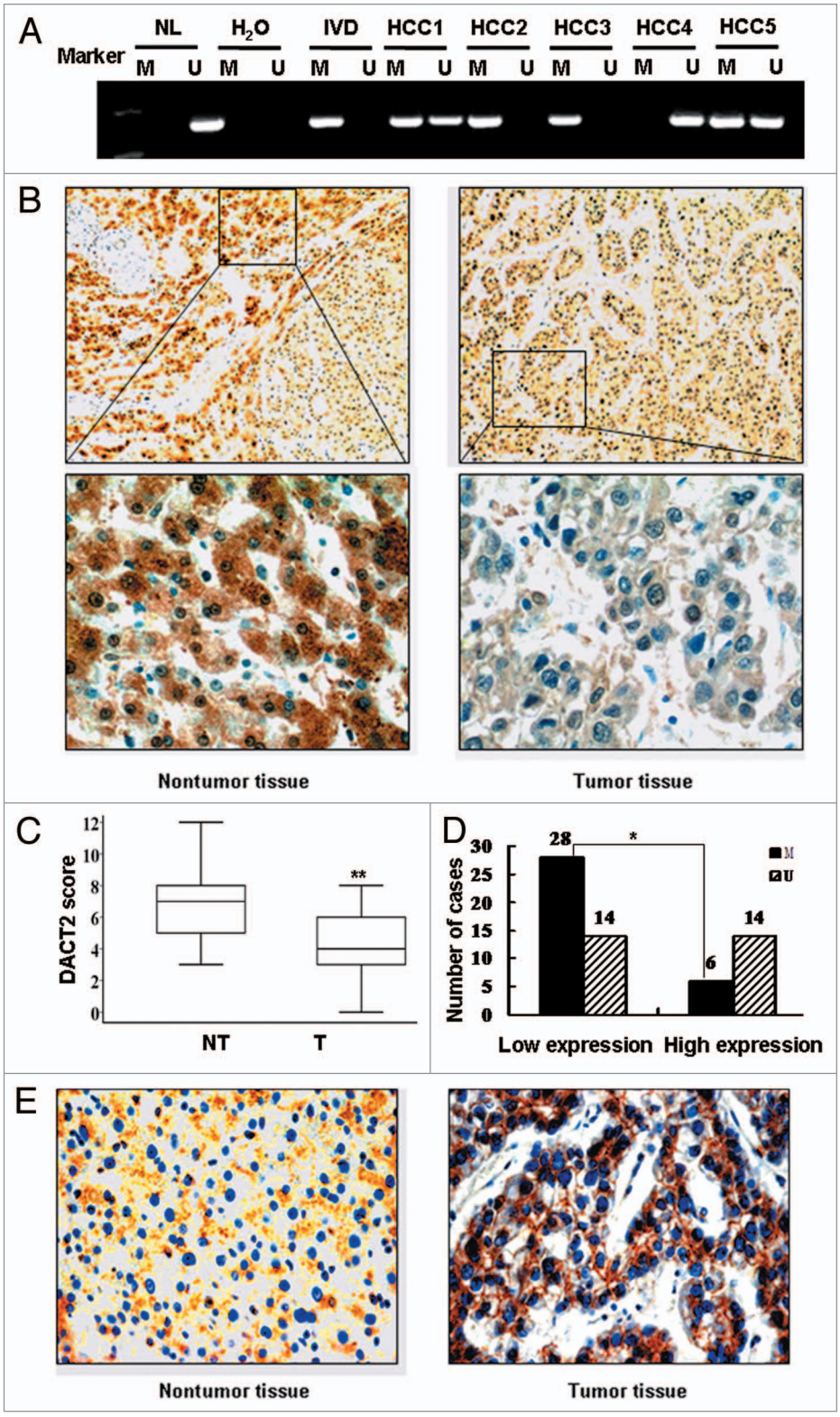

Figure 2. *DACT2* expression is associated with promoter hypermethylation in primary HCCs. (**A**) Representative MSP results of *DACT2* in primary HCCs. (**B**) *DACT2* expression in a representative HCC case. Left image: adjacent non-tumor tissue (100×) and the indicated fields enlarged (400×). Right image: tumor tissue (100×) and the indicated fields enlarged (400×). (**C**) *DACT2* expression scores are shown as box plots, the horizontal lines represent the median score; the bottom and top of the boxes represent the 25th and 75th percentiles, respectively; the vertical bars represent the range of data. Expression of *DACT2* was different between non-tumor tissues and tumor tissues in 62 matched primary HCCs. **$p < 0.01$. (**D**) The correlation of *DACT2* hypermethylation and expression level were analyzed in 62 matched primary HCCs. *$p < 0.05$. (**E**) β-catenin expression in the representative methylated case (400×). β-catenin expression is positive mostly in the membranes of hepatic cells (left image) and in the cytoplasm and nucleus in tumor cells (right image).

DACT genes encode a small family of vertebrate intracellular proteins that can regulate intercellular signaling pathways by a conserved leucine zipper motif near the N-terminus and a binding motif for PDZ domain at the C-terminus.[17,18,31] *DACT1* and *DACT2* were discovered by two independent groups who were screening partners of the Dvl scaffold protein, which is central to the developmentally and clinically important Wnt signaling.[31-36] The initial functional analyses of *DACT2* were based on its overexpression and morpholino-based knockdown technologies in *Xenopus laevis* and zebrafish.[37,38]

In this study, we describe for the first time that *DACT2* is frequently absent or downregulated in HCC cell lines, and is also significantly reduced in primary HCC samples. We first sequenced *DACT2* in primary HCC and cell lines and found no mutation related to HCC. This suggests that genetic changes may not play important roles in HCC carcinogenesis. It was previously reported that *DACT2* is frequently inactivated by DNA methylation in colorectal cancer cell lines (RKO and HT29).[12] Our previous study in lung cancer also reported that the *DACT2* gene was silenced by promoter region methylation.[39] To determine whether hypermethylation was responsible for the silencing of *DACT2* in HCC, promoter region methylation was analyzed in HCC cell lines and primary cancer. Our data suggest that *DACT2* is regulated by promoter region hypermethylation in HCC. Further analysis indicates that reduction of *DACT2* expression was related to younger age and high level of AFP in serum. These results suggest that methylation of *DACT2* may serve as a diagnostic tool and prognostic marker in HCC.

It has been reported that *DACT2* binds to the TGF-β receptors ALK5 and ALK4, accelerating lysosomal degradation of these receptors in zebrafish.[38] Although recent studies showed that murine DACT proteins formed a weak complex with Alk5 in HEK293T cells, no such complex can be detected between Alk4 or Alk5 and *DACT2* protein.[36] *DACT2* strongly co-immunoprecipitated with β-catenin or δ-catenin in HEK293T cells, as well as formed even stronger complexes with CDK1δ/ε, Dvl or Vangl family members.[36] This report suggests that *DACT2* is a Wnt signaling inhibitor. Our study found that HCC proliferation was suppressed by *DACT2* both in vitro and in vivo. In addition, Wnt signaling activity was inhibited by *DACT2* in HCC cells. These results suggest that *DACT2* inhibits human HCC by inactivating Wnt signaling.

In conclusion, *DACT2* is silenced by promoter region hypermethylation in human hepatic cancer and its methylation may serve as a detection marker for HCC. *DACT2* suppressed cell proliferation, induced G2-M arrest in cell cycle and inhibited tumor growth in nude mice. *DACT2* also inhibited *TCF-4* transcriptional activity and its downstream targets (*c-Myc* and *cyclin D1*) in HCC. Based on these findings and its important human chromosomal localization, *DACT2* may be a tumor suppressor in HCC.

Materials and Methods

Ethics statement. For the use of clinical materials for research purposes, prior patients' consent and approval were obtained from Institutional Review Board of the Chinese PLA General Hospital.

Cell lines and tissue samples. Eight HCC cell lines (LO2, SNU182, BEL7402, HepG2, PLC/PRF/5, SMMC7721, SNU449 and 97H) were used in this study. Cell lines were cultured at 37°C in an atmosphere containing 5% CO_2 in 90% RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum. Cells were passaged 1:3 once 80% confluence (approximately 1×10^6 cells) was reached on a 75 cm^2 culture flask (NEST Biotechnology).

Sixty-two paired tumor tissue samples of primary HCC and their adjacent non-tumor tissues were obtained from surgical resected HCC patients at the Chinese PLA General Hospital, Beijing. For this study, all of the tumor and non-tumor tissue were re-examined and confirmed by pathologists.

RNA isolation and semi-quantitative RT-PCR. Total RNA was isolated using the RNeasy mini kit (Qiagen). First strand cDNA (cDNA) was synthesized using the Superscript II-reverse transcriptase kit (Invitrogen). Expression of *DACT2* mRNA (mRNA) was determined by RT-PCR using the LightCycler system (Roche Diagnostics). PCR amplification of *DACT2* was performed using primers: 5'-GGC TGA GAC AAC AGG ACA TCG-3' (forward) and 5'-GAC CGT CGC TCA TCT CGT AAA A-3' (reverse). The primer set for *DACT2* was designed to span intronic sequences between exons in order to control genomic DNA contamination. A total of 35 cycles of amplification were performed for each of the RT-PCR experiments. As an internal control, GAPDH was amplified with 25 cycles to ensure cDNA quality and quantity for each RT-PCR. Amplified products were analyzed on 1.5% agarose gel.

Methylation-specific PCR (MSP) and BSSQ. Genomic DNA from cell lines and tissue samples was prepared using the proteinase-K method. After chloroform/phenol extraction, DNA was precipitated in ethanol and dissolved in low TE buffer and stored at -20°C. Genomic DNA from HCC tissues and cell lines was bisulfite modified as previously described.[40] MSP primers were designed according to genomic sequences flanking the presumed transcription start site (TSS). Primer sequences were oligo-synthesized (Invitrogen) to allow MSP to detect bisulfite-induced changes affecting unmethylated (U) and methylated (M) alleles. MSP of *DACT2* was performed using primers: 5'-GAT TTT AGT TTA TTT TGG CGA TTT GC-3' (M-forward); 5'-CAC ATC TCC CGA ACA AAA TCC CG-3' (M-reverse); 5'-TAG ATT TTA GTT TAT TTT GGT GAT TTG T-3' (U-forward) and 5'- TCC ACA TCT CCC AAA CAA AAT CCC A-3' (U-reverse). Each MSP reaction incorporated approximately 100 ng of bisulfite-treated DNA, 25 pmoles of each primer, 100 pmoles dNTPs, 2.5 μl 10 × PCR buffer, and 1 unit of Taq Polymerase (Invitrogen) in a final reaction volume of 25 μl. Cycle conditions were: 95°C × 5 min, 1 cycle; 35 cycles × (95°C × 30 sec, 60°C × 30 sec, 72°C × 30 sec); 72°C × 5 min, 1 cycle. Each PCR assay included a methylated control (in vitro methylated DNA, IVD), an unmethylated control (normal blood lymphocyte DNA, NL) and a negative control (water). MSP products were analyzed using 2% agarose gel electrophoresis.

Table 1. Association of clinical factors with *DACT2* methylation and expression in HCC patients

Clinical factors	*n*	*DACT2* Methylation (%)	p (χ^2)	Loss of expression (%)	p (χ^2)
Age (years)			1		0.01
< 50	21	12 (57.14)		17 (80.95)	
≥ 50	41	22 (53.65)		18 (43.9)	
Gender			1		0.27
Male	54	30 (55.55)		32 (59.26)	
Female	8	4 (50.00)		3 (75.00)	
Virus infection			0.6		0.27
HBV	49	28 (57.14)		28 (57.14)	
HCV	2	1 (50.00)		2 (100.00)	
No	11	5 (45.45)		5 (45.45)	
AFP (ng/ml)			1		0.04
< 500	44	24 (54.54)		21 (47.73)	
≥ 500	18	10 (55.55)		14 (77.78)	
Tumor size (cm)[a]			0.79		0.7
< 8.07	36	19 (52.77)		21 (58.33)	
≥ 8.07	26	15 (57.69)		14 (53.84)	
ALTSG stage			1		1
T1,T2	15	8 (53.33)		8 (53.33)	
T3,T4	47	26 (55.32)		27 (57.44)	
AJCC stage			0.17		0.78
T1,T2	36	24 (66.67)		24 (66.67)	
T3,T4	21	10 (47.62)		11 (52.38)	
Differentiation			0.96		0.67
Poor	10	6 (60.00)		6 (60.00)	
Poor to moderate	8	4 (50.00)		3 (37.50)	
Moderate	36	20 (55.56)		20 (55.56)	
Moderate to well	3	2 (66.67)		2 (66.67)	
Well	5	2 (40.00)		4 (80.00)	
Cirrhosis			0.77		0.57
No	16	8 (50.00)		8 (50.00)	
Yes	46	26 (56.52)		27 (58.70)	
Capsule invasion[b]		0.315			0.1
No	39	20 (51.28)		25 (64.10)	
Yes	20	13 (65.00)		8 (40.00)	
Encapsulated[b]			1		0.30
No	12	6 (50.00)		5 (41.67)	
Yes	31	16 (51.61)		20 (64.52)	
Vascular invasion			0.54		0.53
No	49	28 (57.14)		29 (59.18)	
Yes	13	6 (46.15)		6 (46.15)	

[a]The average of tumor size of all 62 samples is 8.07 cm. [b]Information on capsule invasion and encapsulation is only available for 59 and 43 samples, respectively.

Bisulfite-treated DNA was subjected to PCR using primers flanking the targeted MSP regions above. Sequencing primers were as follows: 5'-TGG TTA TAG ATT TTA GTT TAT TTT GG-3' (forward) and antisense 5'-CAA CCC CTA CAA CTC CTA CAA C-3' (reverse). PCR cycle conditions were as follows: 95°C × 5 min, 1 cycle; 35 cycles × (95°C × 30 sec, 58°C × 30 sec, 72°C × 40 sec); 72°C × 5 min, 1 cycle. PCR products were gel purified and cloned into pCR2.1 vector according

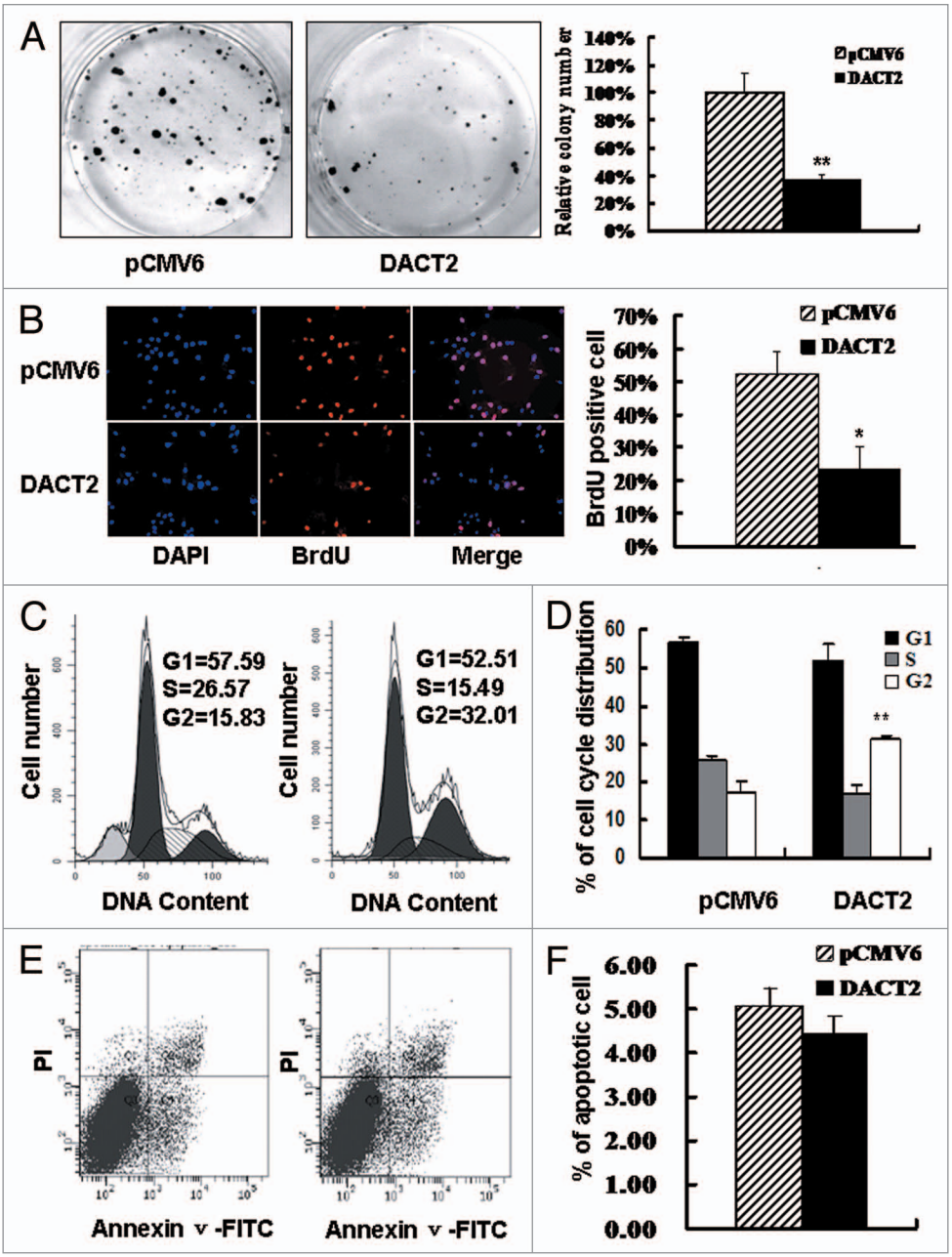

Figure 3. The effect of *DACT2* on cell proliferation, cell cycle and apoptosis in vitro. (**A**) The effect of *DACT2* on HCC cell proliferation was evaluated by colony formation assay. Left panel: Colony formation results of transfected with pCMV6 (empty vector) or *DACT2* expression vector in HepG2 cells. Right panel: Quantitative analysis of colony numbers in different group. **p < 0.01. (**B**) BrdU incorporation was analyzed in pCMV6 or *DACT2* expression vector transfected HepG2 cells. Left panel: cell nuclei were counterstained by BrdU and DAPI. Right panel: quantitative analysis of BrdU positive cells in different group. *p < 0.05. (**C**) Representative results of cell cycle distribution for pCMV6 (left panel) or *DACT2* expression vector (right panel) transfected in HepG2 cells. Cycle distributions were measured by propidium iodide (PI) staining followed by flow cytometry after transfection for 48 h. (**D**) Quantitative analysis of cell cycle distribution in *DACT2* expression and control group. **p < 0.01. (**E**) No effect was found on apoptosis after expression of *DACT2* in HepG2 cell line. HepG2 cells were transfected with pCMV6 (left panel) or *DACT2* expression vector (Right panel). 48 h serum starvation was performed 24 h after transfection. Then Annexin V-FITC/PI double staining was performed. Annexin V-positive and PI-negative staining cells are indicated as apoptotic cells. (**F**) Quantitative analysis of cell apoptosis.

to the manufacturer's instructions (Invitrogen). Sequencing was performed as previously reported.[5,41]

5-aza-2'-deoxycytidine (5-AZA) treatment. Cell lines (HepG2, SNU182, BEL7402, PLC/PRF/5 and LO2) were split to a low density (30% confluence) 12 h before treatment. Cells were treated with 5-AZA (Sigma) at a concentration of 2 μM. Growth medium, conditioned with 5-AZA at 2 μM, was exchanged every 24 h for 96 h. At the end of the treatment, RNA was extracted from the cells as described above.

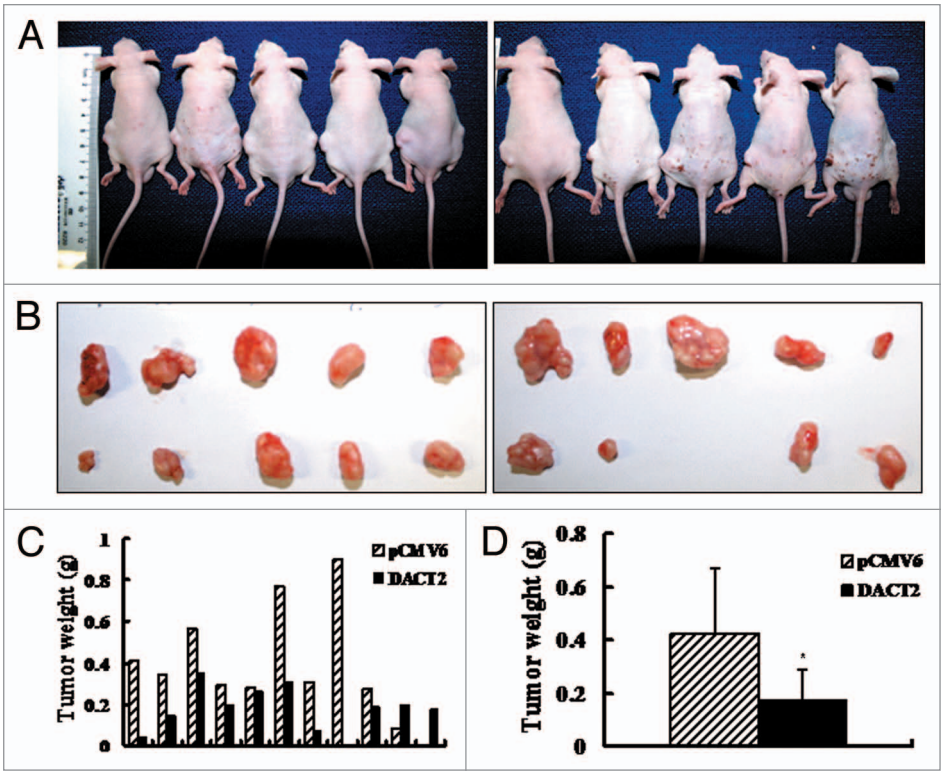

Figure 4. Tumor growth was inhibited by *DACT2* in vivo. (**A**) Nude mice inoculated with HepG2 cells expressing or not expressing *DACT2*. Left rear flank: empty vector group. Right rear flank: *DACT2*-expressing group (n = 10/group). (**B**) Tumors in the upper panel are from the empty vector group and tumors in the lower panel are from the *DACT2*-expressing group. (**C**) Histogram represents tumor weights of each mouse in the two groups. (**D**) Tumor weights are significantly different between *DACT2*-expressing and empty vector groups. *p < 0.05.

Immunohistochemistry staining. Rabbit anti-*DACT2* antibody (Prosci) and mouse anti-β-catenin antibody (ZSGB Biotech.) were employed. Immunohistochemistry (IHC) was performed on 4 μm thick serial sections derived from formaldehyde fixed paraffin wax embedded tumor tissue blocks. After deparaffinization and rehydration, endogenous peroxidase activity was blocked for 30 min in methanol containing 0.3% hydrogen peroxide. The slides were then incubated with anti-*DACT2* antibodies (1:1600 dilution) or with mouse anti-β-catenin antibody (1:400) overnight at 4°C in a humidified chamber. IHC was performed on the tissue sections according to the Polink-2 plus® Polymer HRP Detection System (ZSGB Biotech). Hematoxylin was used for counterstaining. The expression of *DACT2* and β-catenin was evaluated according to previous report.[42]

Construction of *DACT2* expression vector and isolation of *DACT2* expressed cells. The expression construct for *DACT2* was generated by cloning a PCR-amplified full-length human *DACT2* cDNA fragment (GenBank accession number NM_214462) into pCMV6-AC-GFP or pCMV6-Entry-myc (Origene Technologies, Inc.). The vector was verified by DNA sequencing and western Blotting. Transient transfection was performed using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. GFP positive cells were isolated by flow cytometry (Becton Dickinson) 48 h after transfection.

Western blotting. Cell lysates were collected and western blotting was performed. The antibodies used included rabbit anti-*DACT2* antibodies (Prosci) and monoclonal antibodies against cyclin D1 (Santa Cruz Biotechnology) as well as c-Myc and actin (Bioworld Technology).

Colony formation assay. HepG2 cells were transfected with either an empty or a *DACT2* expression vector using FuGENE HD (Roche Applied Science) according to manufacturer's instructions. 60% GFP positive cells were isolated by flow cytometry 48 h after transfection. Cells were collected and reseeded in triplicate at 1,500 cells per well in 6-well plates. Growth medium, conditioned with G418 (Invitrogen) at 500 μg/mL, was exchanged every 24 h. After 10–14 d, cells were fixed with 75% ethanol for 30 min, stained with 0.2% crystal violet for visualization, and counted.

BrdU incorporation analysis. HepG2 cells, transfected with either an empty or a *DACT2* expression vector, were seeded in 24-well plates. Ten milimolar BrdU was added in growth medium and incubated at 37°C for 2 h. Cells were stained with mouse anti-BrdU antibody and DAPI for double labeling. The BrdU (red) /DAPI (blue) positively staining cells are indicated as positive BrdU incorporation.

Cell cycle analysis. DACT or empty vector was transfected into HepG2 cells using FuGENE. Forty-eight hours after transfection, cells were harvested, washed with phosphate-buffered saline (PBS) and fixed with ice-cold 70% ethanol at -20°C overnight. Samples were then washed with PBS and stained with propidium iodide (Sigma) containing RNase A (Sigma) for 30 min at 37°C. Cell cycle distribution in different phases was determined using flow cytometry.

In vivo tumor genesis assay. Six-week-old female nu/nu mice were bred under specified pathogen-free conditions. HepG2 cells were transiently transfected with either an empty or a *DACT2* expression vector using FuGENE HD and GFP positive cells were isolated by flow cytometry 48 h after transfection. Cells (3×10^6) were diluted in 100 μl PBS and injected subcutaneously into the left or right rear flank of the mice. When tumors reached approximately 1.0 cm in size, the mice were sacrificed and the volume as well as the weight of the tumors were measured. Tumor volume (mm^3) was estimated by measuring the longest and shortest diameter of the tumor and calculated with the formula: tumor volume = (length) × $(width)^2/2$. All experimental procedures were approved by the Animal Ethics Committee of the Chinese PLA General Hospital, Beijing.

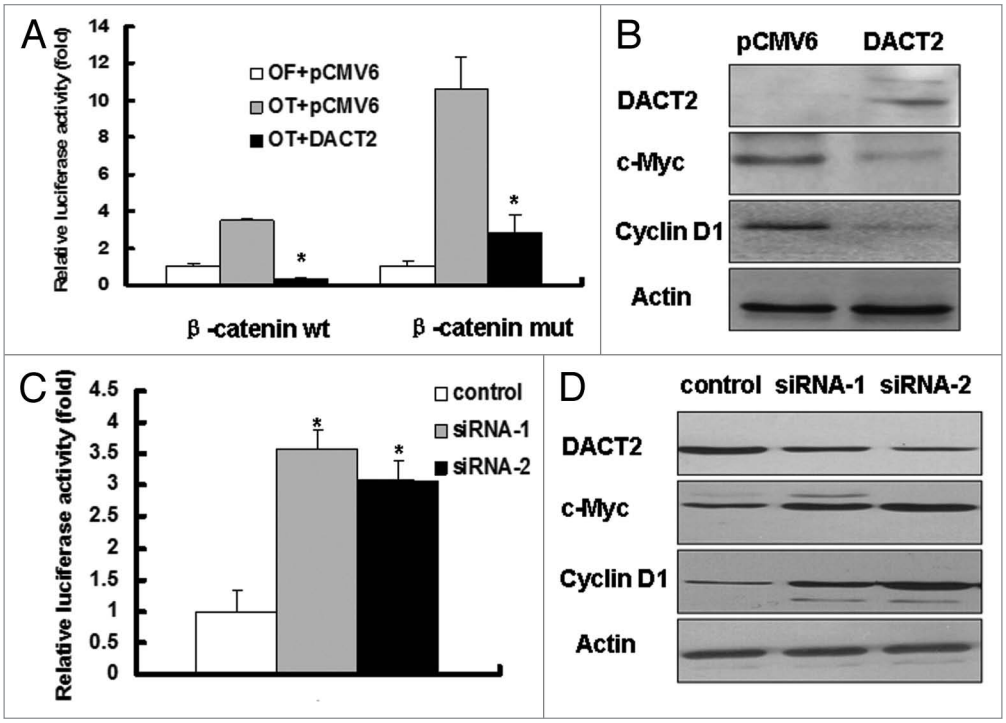

Figure 5. *DACT2* is a Wnt signaling inhibitor. (**A**) The transcriptional activity of *TCF-4* was inhibited by *DACT2*. Left panel: *DACT2* was co-transfected with wt β-catenin and OT into HepG2 cells. Right panel: *DACT2* was co-transfected with mut β-catenin and OT into HepG2 cells. OF acted as a negative control reporter and its transcriptional activity was defined as 1. *$p < 0.05$. (**B**) Expression of c-Myc and cyclin D1 was analyzed by western blot in *DACT2*-expressing or -not expressing HepG2 cells. Actin was used as an internal control. (**C**) The transcriptional activity of *TCF-4* was increased after two individual siRNAs targeting *DACT2* vectors were transfected into PLC/PRF/5 cells. *$p < 0.05$. (**D**) Knockdown of *DACT2* increases the expression of c-Myc and cyclin D1 in PLC/PRF/5 cells. Actin was used as internal reference.

Dual-luciferase reporter assay. To explore the effect of *DACT2* on Wnt signaling, *DACT2* was co-transfected with TCF-4 reporter, pTOPFlash (OT), and β-catenin wide type (wt) or β-catenin mutant (mut) vector into HepG2 cells, pFOPFlash (OF) acted as a negative control reporter.[43] Cell lysates were collected and luciferase enzymatic luminescent activity was measured 48 h after transfection according to the manufacturer's instructions (GLOMAX luminometer, Dual Luciferase Reporter Assay system, Promega).

RNA interference. Two selected siRNAs targeting *DACT2* and RNAi Negative Control Duplex were used in this study. The sequences are as follows: siRNA-1 (sense sequence: 5'-CCA GCU GUC CUG AGU CUA ATT-3' and antisense sequence: 5'-UUA GAC UCA GGA CAG CUG GTT-3'); siRNA-2 (sense sequence: 5'-GUC GGU UGA UGA GAC UAC UTT-3' and antisense sequence: 5'-AGU AGU CUC AUC AAC CGA CTT-3'). RNAi Negative Control Duplex (sense sequence: 5'-UUC UCC GAA CGU GUC ACG UTT-3'; and antisense sequence: 5'-ACG UGA CAC GUU CGG AGA ATT-3'). Fifty to sixty percent confluent PLC/PRF/5 cells were transfected with 50 nM of siRNAs using Lipofectamine 2000 (Invitrogen) following the manufacturer's direction.

Statistical analysis. We evaluated the relationship between methylation status in human hepatocellular carcinomas and clinicopathologic characteristics using the Pearson's chi-square test or the Fisher's exact test for independence for dichotomous variables as appropriate. Continuous variables were analyzed with Student's t-test. Results were judged to be statistically significant at $p < 0.05$ and all p values were two-sided. All analyses were done using SPSS PASW Statistics 18.0.

Disclosure of Potential Conflicts of Interest

J.G.H. is a consultant to MDxHealth. The other authors declare no conflict of interest.

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