

Research Paper

Epigenomic and Functional Characterization of Junctophilin 3 (JPH3) as a Novel Tumor Suppressor Being Frequently Inactivated by Promoter CpG Methylation in Digestive Cancers

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Abstract

Junctophilin (JPH) proteins stabilize junctional membrane complexes between plasma membrane and endoplasmic reticulum, also implicated in some human diseases. *JPH3* mutations are linked to Huntington's disease-like 2 syndrome. Through epigenomic study of a colon cancer cell line pair (HCT116 and DKO), we identified *JPH3* as a methylated novel tumor suppressor gene (TSG) candidate at 16q24. We further studied its epigenetic alterations and functions in digestive tumorigenesis. *JPH3* expression at the RNA level was found to be frequently silenced or reduced in colorectal and gastric cancers due to its promoter CpG methylation, which is associated with tumor progression and poor survival of digestive cancer patients. Ectopic expression of *JPH3* inhibited tumor cell growth *in vitro* and *in vivo*. JPH3 expression upregulated the cytosolic Ca²⁺ levels, and unfolded protein response gene expression upon endoplasmic reticulum stress. JPH3 also induced calpain activation and subsequent mitochondrial membrane depolarization and cell apoptosis. Thus, *JPH3* was identified as a novel TSG methylated in colorectal and gastric tumors which promotes mitochondrial-mediated apoptosis, also as a potential metastasis and survival biomarker for digestive cancers.

Key words: junctophilin, tumor suppressor gene; methylation; metastasis, 16q24.

Introduction

Gastric and colorectal cancers remain the third and fourth most common cancers and the second and third most common causes of cancer mortality worldwide. The gradual accumulation of multiple genetic and epigenetic changes genome-wide is the molecular basis of their initiation and progression [1]. Epigenetic modifications, particularly promoter

CpG methylation, play a key role in their morphological progression. DNA methylation often results in the hypermethylation of selected gene promoters and inactivation of tumor suppressor genes (TSGs) [2]. Epigenetic disruption of TSGs is a fundamental contributor to digestive tumor development. Thus, identification of novel

hypermethylated genes offers molecular insights into potential screening, monitoring, and therapeutic strategies to digestive cancers [3-5].

16q24 is a frequently deleted TSG locus in multiple tumors, including cancers of digestive, nasopharyngeal, breast, prostate and liver tissues. Loss of heterozygosity (LOH) at 16q24 is also frequently present in multiple other solid tumors, such as breast, prostate, gastric, colorectal, hepatocellular and Wilm's tumor [6-10], suggesting the presence of critical TSG(s) at this locus. Several *bona fide* TSGs have been identified at this locus including *CDH13* [11]. We previously also used integrative genomics/epigenetics to identify TSGs at this critical locus and have successfully identified *IRF8* [12], *CDH11* [13]. We propose that more critical undocumented TSGs may reside at this locus, which needs to be further investigated.

Junctophilins (JPHs) belong to members of the junctional membrane complex (JMC) protein family, serving to stabilize junctional membrane complexes between endoplasmic reticulum (ER) and plasma membrane (PM), as well as maintain cellular ultrastructure between the cell surface and intracellular ionic channels. JPHs are essential for proper Ca^{2+} -induced Ca^{2+} release during the excitation-contraction coupling. Four JPH family members, JPH1-JPH4, have been identified [14, 15] with highly conserved across species and tissue-specific expression manner. Abnormalities associated with JPH proteins have been linked to human diseases [16], including cardiomyopathy, arrhythmia, heart failure, skeletal muscle myopathy and Huntington's disease (HD)-like pathology. For an example, in Huntington's disease-like 2 (HDL2), decreased *JPH3* transcripts and proteins are detected in HDL2 brain, probably due to the abnormal CAG/CTG repeat expansions at the HDL2 locus involving a junctional complex protein JPH3, while JPH3 hemizygous or null mice display impaired motor function [16, 17]. However, the association of alterations and functions in JPH family proteins with human tumorigenesis has been rarely studied.

In this study, we searched for methylated TSG candidates for digestive cancers through epigenomic (CpG methylome) study and expression profiling of a paired colon cancer cell line (HCT116 and HCT116-DKO with double knock-out of *DNMT1* and *DNMT3B* [18, 19]), and identified a candidate 16q24 TSG - Junctophilin-3 (*JPH3*) as a methylated target. We systematically evaluated the inactivation of *JPH3* by promoter CpG methylation in colorectal and gastric cancers, further explored its functions and mechanisms in the development of these cancers.

Results

Epigenomic identification of *JPH3* as a methylated target in digestive cancers

Through analyzing whole-genome CpG methylation profiles (methylomes) of a paired colon cell line, HCT116 and DKO (with double knock-out of *DNMT1* and *DNMT3B*), we found a significant signal enrichment of CpG methylation (cut off = 2) at the *JPH3* promoter and exon 1 region in HCT116, but not in DKO (Figure 1A), suggesting that *JPH3* is a methylated target in colorectal cancer.

We next examined *JPH3* mRNA and protein expression in human normal adult tissues by semi-quantitative RT-PCR and immunohistochemistry. Results showed that in addition to its expected abundant expression in brain, *JPH3* was also expressed in most other normal tissues including digestive tissues such as stomach, colon and rectum (Figure 1B). *JPH3* is expressed in the cytoplasm of normal colonic and gastric mucosa epithelium (Figure 1B). We then examined its expression in colorectal and gastric tumor cell lines and found that *JPH3* was silenced or dramatically reduced in most cell lines (Figure 1D, Suppl. Figure S1, and S3).

As *JPH3* has 4 splicing variants, with only variant 1 as the longest full-length transcript containing 5 coding exons and encoding a fully functional 748-amino acid protein; while variant 2 and 3 are much shorter isoforms with only two coding exons and distinct C-termini, and variant 4 is actually a non-coding RNA. We designed more RT-PCR primers to examine the expression of these variants in cell lines and some primary tumors (Figure 1, Suppl. Figure S1, Suppl. Table S1). We found that variant 1, 4, 2 were frequently downregulated or silenced in most colorectal (CRC) and gastric cancer cell lines and primary tumors, while no expression of variant 3 was detected in any CRC and gastric tumor cell line or tumor (Figure 1, Suppl. Figure S1).

JPH3 contains a typical CpG island (CGI) spanning its promoter and exon 1 (Figure 1C). Methylation-specific PCR (MSP) was used to analyze its methylation status and found that the *JPH3* CGI was methylated in all cell lines with silenced or reduced expression (Figure 1D). *JPH3* expression was restored after demethylation treatment with 5-Aza and TSA in methylated and silenced cell lines (Figure 1E). We further examined the detailed methylation profiles by bisulfite genomic sequencing (BGS) analysis of 81 CpG sites of the CGI, including sites analyzed by MSP (Figure 1F). Densely methylated CpG sites were detected in silenced cell lines, while both MSP and BGS showed that the *JPH3* CGI was

demethylated after 5-Aza and TSA treatment (Figure 1E, 1F), revealing a link between CGI methylation and *JPH3* silencing. We also examined *JPH3* expression and methylation in DKO cells. Results showed that *JPH3* expression was restored in DKO cells, together with completely demethylated promoter alleles, suggesting that *JPH3* transcription is regulated by promoter CpG methylation (Figure 1E).

JPH3 inhibited the growth and migration of digestive tumor cells

Frequent *JPH3* silencing by promoter methylation in colorectal and gastric cancers suggests that *JPH3* is likely a tumor suppressor. We investigated the effect of ectopic *JPH3* expression on digestive tumor cell growth and migration. *JPH3* expression plasmid was stably transfected into

methyated and silenced HCT116 colon and MKN-28 gastric tumor cells. Ectopic *JPH3* expression significantly suppressed the colony formation efficiency in plate and soft agar of transfected cells, relative to both vector-transfected cells and control cells ($p < 0.05$, Figure 2A, 2B), indicating that *JPH3* possesses growth inhibitory activities in colorectal and gastric cancer cells and functions as a tumor suppressor. Re-expression of *JPH3* in these cells was confirmed by RT-PCR and Western blot (Figure 2C, Suppl. Figure S3). We evaluated the effects of *JPH3* on the growth of MKN-28 cells in nude mice *in vivo*. The growth curve of *JPH3* stably transfected and vector-transfected MKN-28 cells in nude mice was shown in Figure 2D, with no tumor grown in the *JPH3*-expressing cell group.

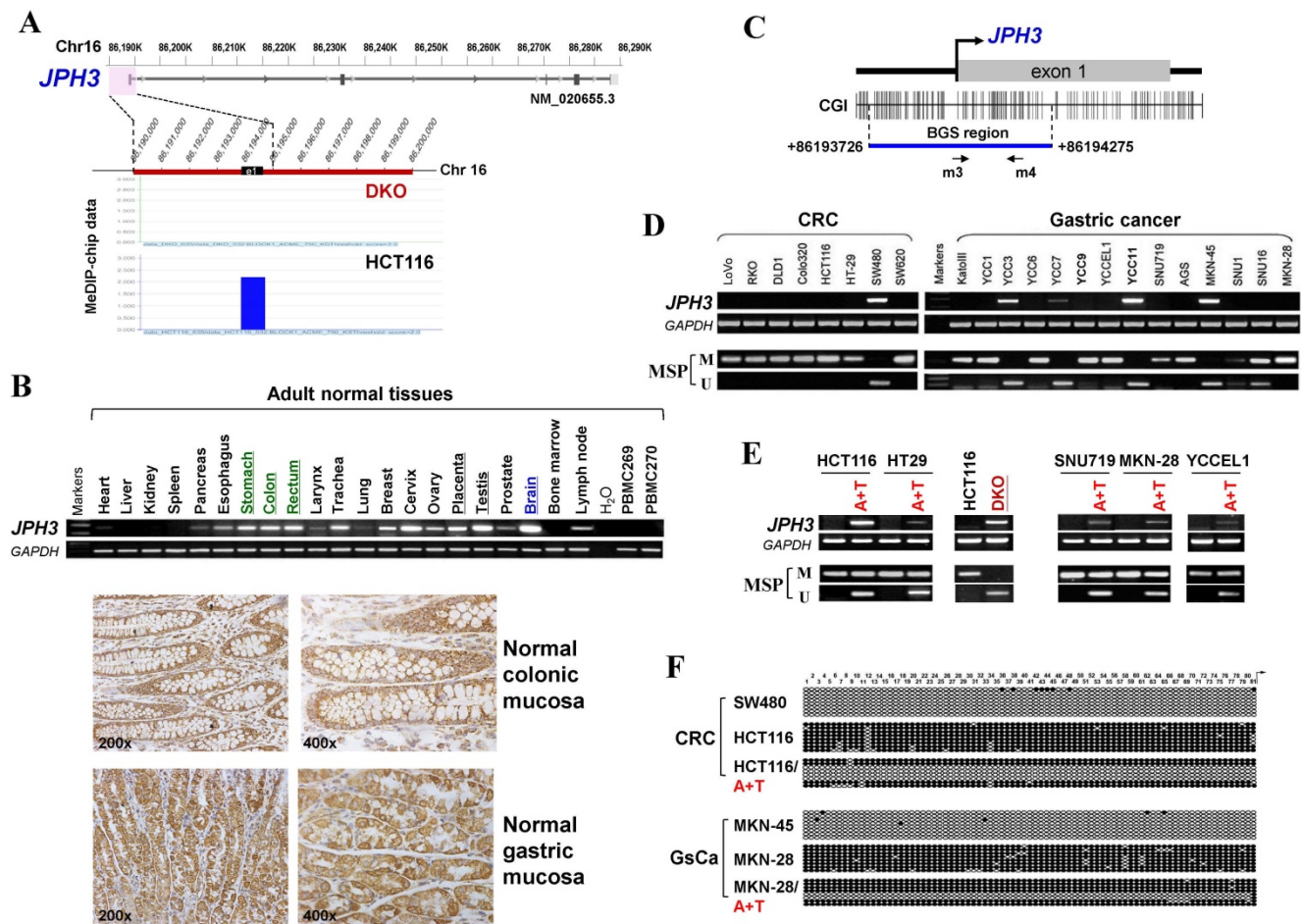


Figure 1. *JPH3* expression and methylation in colorectal and gastric cancer cell lines and primary tumors. (A) CpG methylome study identified *JPH3* as a methylated target in HCT116. *JPH3* gene structure, promoter and exon 1 (UCSC Genome Browser NCBI36/hg18) are shown on the top panel. e1: exon 1. Positive methylation signal peak (blue) in HCT116 was identified by MeDIP-chip. (B) Broad expression of *JPH3* in a normal adult tissue panel by semi-quantitative RT-PCR (primers detecting both variant 1 and 4), with *GAPDH* as a control (upper). Representative immunohistochemical staining for *JPH3* in normal colonic and gastric mucosa. Original magnification: 200x, 400x (lower). (C) Structure of the *JPH3* promoter CpG island (CGI). CpG sites are shown as short vertical lines. MSP primer sites and BGS region analyzed are also indicated. (D) Downregulation and silencing of *JPH3* in colorectal and gastric cancer cell lines as a result of its CGI methylation, with RT-PCR primers detecting both variant 1 and 4. (E) Pharmacological demethylation with 5-Aza and TSA restored *JPH3* expression in methylated and silenced cell lines. Representative results are shown. (F) Representative BGS results of cell lines. Vertical lines indicate individual CpG sites. Cloned BGS-PCR products were sequenced and each colony is shown as an individual row, representing a single allele of the CGI. Filled circles represent methylated and open circles unmethylated CpG sites. U: unmethylated; M: methylated. A: 5-Aza; T: TSA; CRC, colorectal cancer; GsCa, gastric cancer.

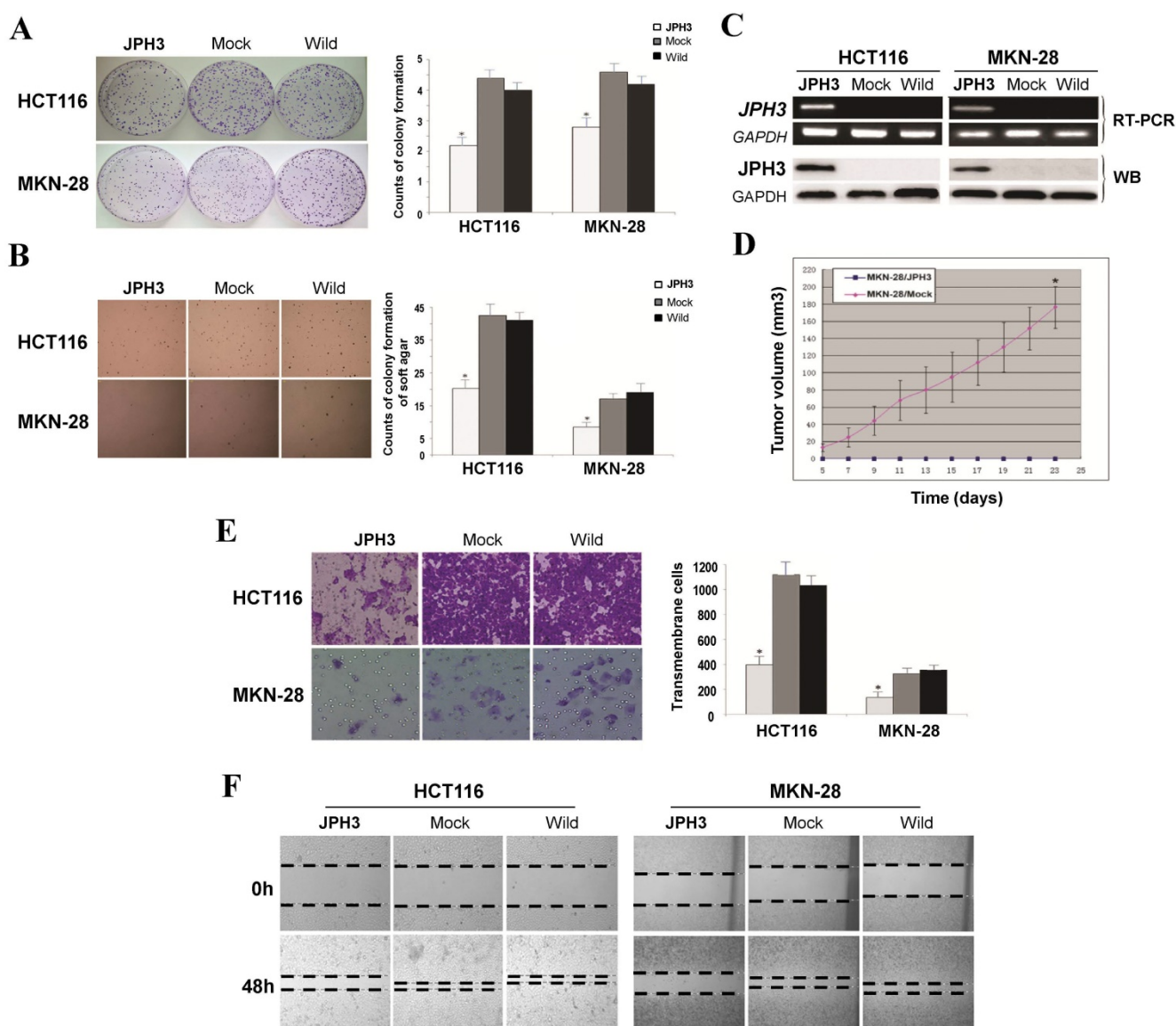


Figure 2. JPH3 inhibited the growth and migration of cancer cells. (A) Representative colony formation assay by monolayer culture and quantitative analysis. (B) Representative results of soft agar assays and quantitative analysis. (C) JPH3 expression in stably transfected cells confirmed by RT-PCR and Western blot. (D) Effect of JPH3 expression on tumor growth in nude mice. Result of tumor growth in nude mice inoculated subcutaneously with MKN-28/vector or MKN-28/JPH3 cells. *: $p < 0.05$. (E) Migration assay with a 24-transwell system and quantitative analysis. The pictures were taken 24 h after seeding. Original magnification: 100x. (F) Wound-healing assay. Photos were taken every 24 h. Original magnification: 100x. All values are the mean \pm s.d. of three independent experiments. *: $p < 0.05$.

To further evaluate the effects of JPH3 expression on the migration and invasion of colorectal and gastric cancer cells, transwell migration and wound-healing assays were performed. JPH3 expression also inhibited cancer cell migration and invasion significantly ($p < 0.05$, Figure 2E, 2F). These results suggested that JPH3 indeed acts as a tumor suppressor in colorectal and gastric cancer cells through inhibiting cell growth and migration.

JPH3 promoted ER stress-induced apoptosis and unfolded protein response

To further determine the mechanisms of JPH3-mediated tumor inhibition, we quantified cell cycle distribution using flow cytometry. There were

no significant differences in cell cycle phase distribution between JPH3-transfected and control cells. We further checked spontaneous, 5-fluorouracil and ER stress-induced apoptosis by inducer brefeldin A (BFA) treatment. JPH3 expression increased ER stress-induced apoptosis (Figure 3A) but not the spontaneous and 5-fluorouracil induced apoptosis. JPH3 expression also increased caspase 3, 9 and PARP cleavage which was further enhanced by BFA treatment (Figure 3B). Although caspase 4 activation has been reported to be responsible for ER stress-mediated apoptosis, our data indicate that JPH3 expression-elicited apoptosis might be caspase 4-independent (Figure 3B).

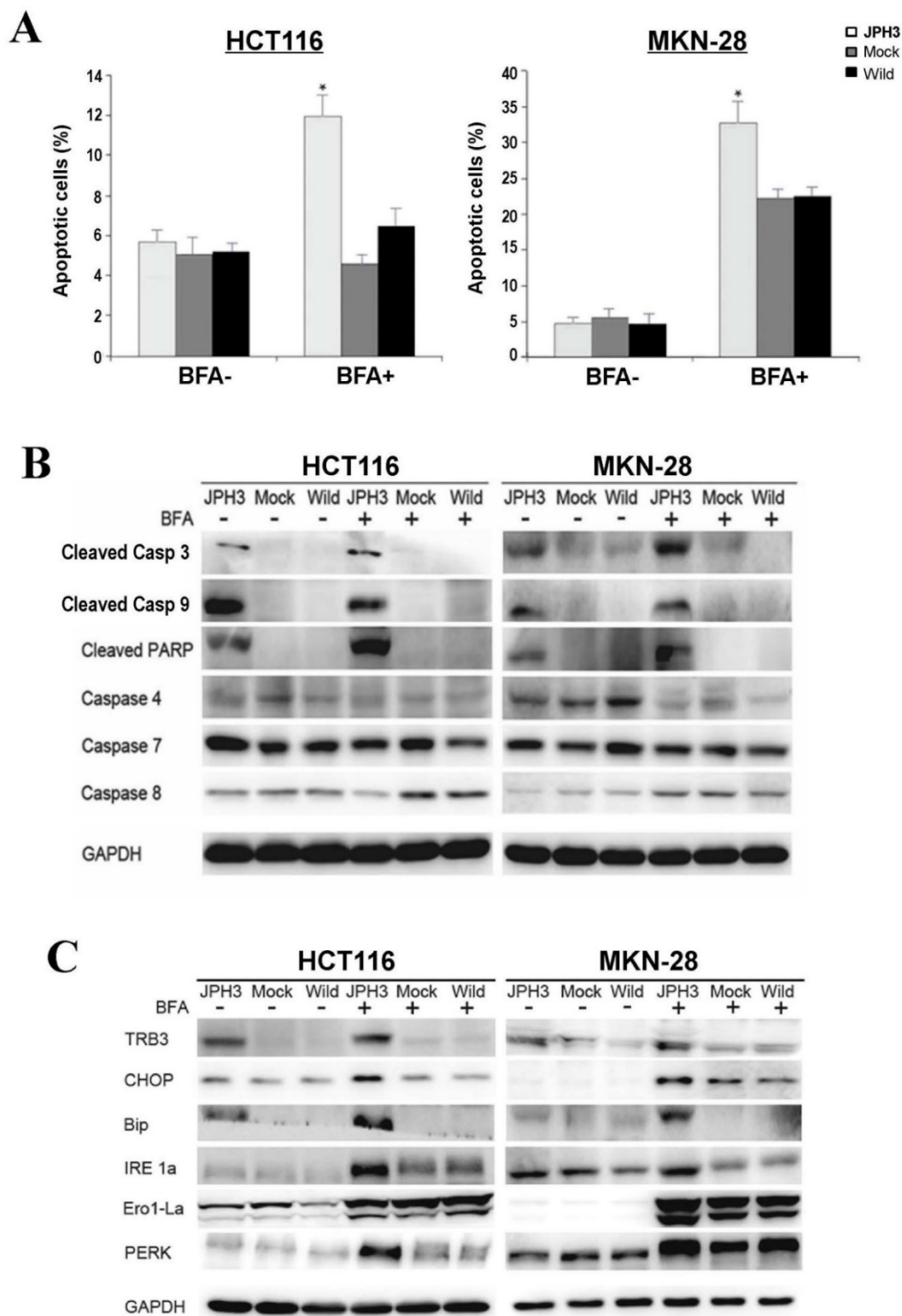


Figure 3. Effects of ectopic JPH3 expression on the apoptosis and UPR of tumor cells with or without BFA treatment. (A) Quantitative analysis results of annexin V-FITC/PI staining before and after BFA treatment in HCT116 and MKN-28 cells. Values are the mean \pm s.d. of three independent experiments. *: $p < 0.05$. (B) Cleaved Caspase 3, Caspase 9 and PARP were significantly induced by JPH3 expression. (C) Expression of several key UPR markers examined by Western blot.

In addition, we assessed the effects of JPH3 on tumor suppressor p53, a key regulation of cell cycle arrest and apoptosis. Immunofluorescence and WB results showed no difference in p53 protein levels

between JPH3 expression cells and their controls (Suppl. Figure S4).

To determine the effect of JPH3 expression on unfolded protein response (UPR), the expression of

some UPR-related proteins was examined. As shown in Figure 3C, JPH3 expression upregulated several UPR-related proteins, including TRB3, CHOP, Bip, IRE1a, Ero1-La, and PERK, especially after BFA treatment. These results suggested that JPH3 could increase ER stress-induced apoptosis and UPR in colorectal and gastric cancer cells.

JPH3 regulated the mitochondrial apoptotic signaling pathway

We next investigated whether JPH3 affects mitochondrial apoptotic signaling pathway. Our results show that JPH3 expression could induce collapse of the mitochondrial transmembrane potential (MMP) even before BFA treatment (Figure 4A). To determine whether the MMP change led to release of mitochondrial apoptogenic factor Cyto c and Smac, the cytosolic and mitochondrial fractions were analyzed by immunoblotting. Results showed that JPH3 expression increased the release of Cyto C and Smac into the cytosol (Figure 4B).

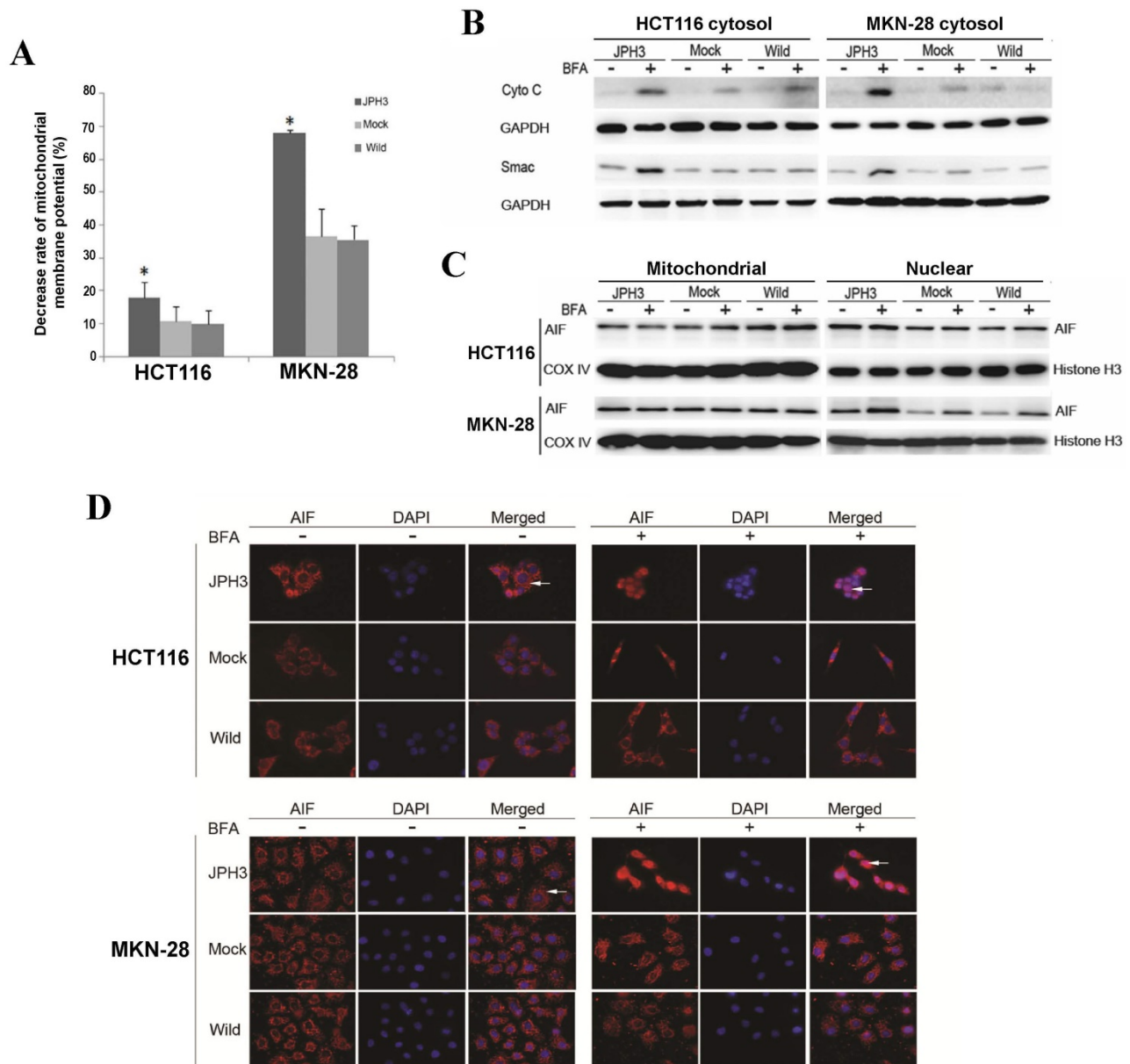


Figure 4. Effects of ectopic JPH3 expression induced digestive cancer cell apoptosis through mitochondria-mediated apoptosis pathway. (A) Mitochondrial membrane potential was significantly decreased by JPH3 expression. (B) JPH3 expression increased the release of Cyto C and Smac into the cytosol. (C) Western blot and (D) immunofluorescence results showed that JPH3 expression induced translocation of mitochondrial AIF into the nucleus significantly after BFA treatment.

To further determine whether another mitochondrial apoptogenic factor, AIF, was also involved in JPH3 expression induced apoptosis, Western blot and immunofluorescence experiments were performed to confirm the nuclear translocation of AIF (Figure 4C, 4D). After BFA treatment, AIF colocalized with DAPI nuclear stain in most JPH3 expressing cells, while in control cells AIF staining was always observed in the cytosol (Figure 4D). Meanwhile, Western blot showed that AIF expression increased in nuclear fraction but decreased in mitochondrial fraction, especially after BFA treatment (Figure 4C). These data suggested that JPH3 is involved in modulation of the mitochondrial

apoptotic signaling pathway in colorectal and gastric tumor cells.

JPH3 expression increased cytosolic Ca²⁺ level and calpain activation

As previous studies reported that JPHs are essential for the maintenance of JMC structures and thus participate in the maintenance of intracellular Ca²⁺ homeostasis and regulation of intracellular calcium signaling pathways. We also analyzed the effect of JPH3 expression on cytosolic Ca²⁺ levels. JPH3 expression increased the cytosolic Ca²⁺ level to about twice that of the controls (Figure 5A, 5B).

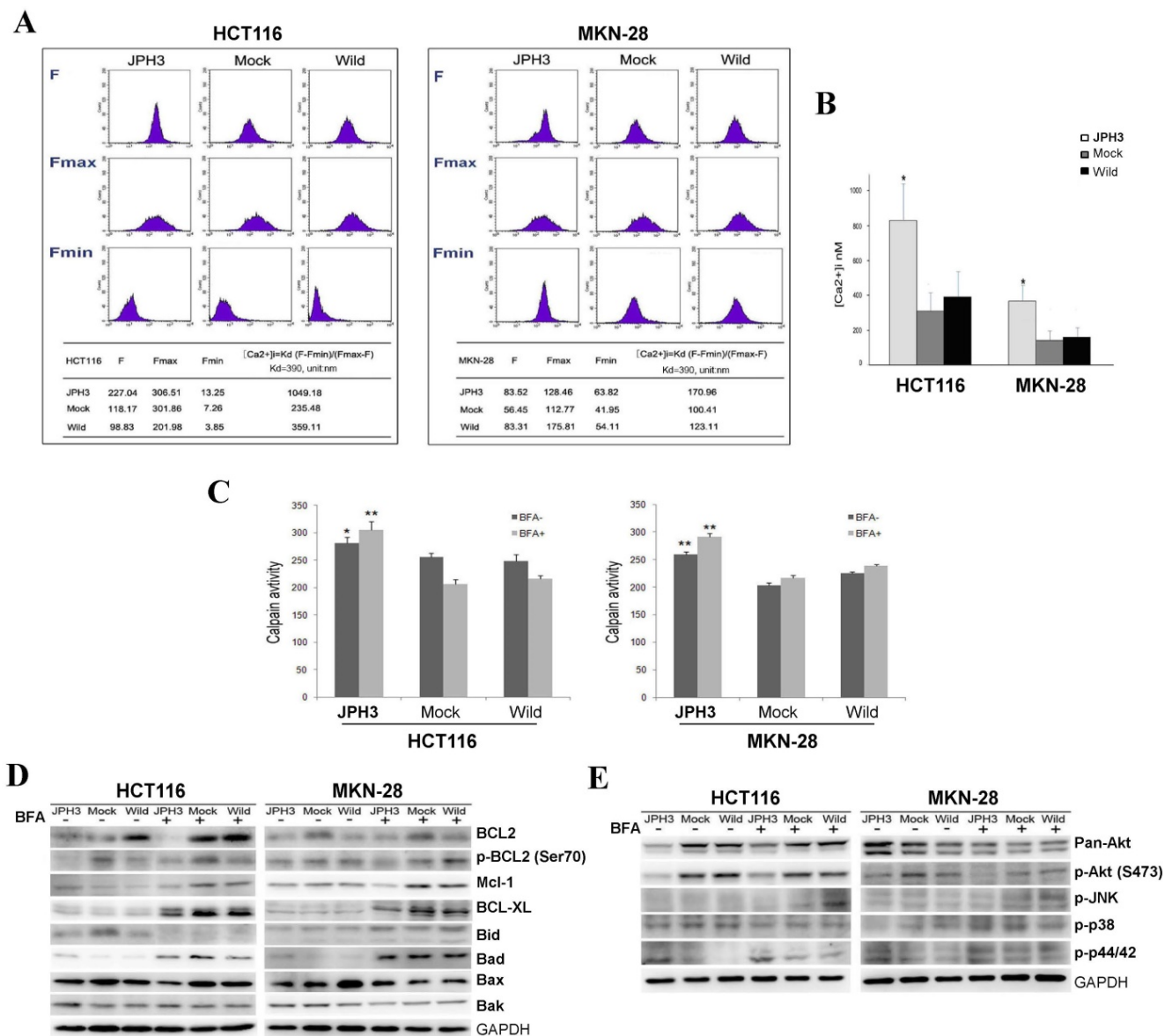


Figure 5. Effects of ectopic JPH3 expression on the level of cytosolic Ca²⁺, Calpain activity, several important protein kinases and BCL2 family protein expression. (A-B) JPH3 expression significantly increased the level of cytosolic Ca²⁺ and (C) Calpain activity. (D) JPH3 expression significantly decreased the pro-survival Bcl2 family proteins including Bcl2, P-Bcl2 (Ser70), Mcl-1 and Bcl-xL, but had no apparent effect on the pro-apoptosis Bcl2 family proteins. (E) JPH3 expression significantly decreased p-Akt (S473) expression and had no apparent effects on the others.

To further define the Ca²⁺-mediated signaling pathway, we examined whether calpain was activated by JPH3 expression. Calpain activity was significantly higher in JPH3 expressing cells than in the controls especially after BFA treatment (Figure 5C). These results suggested that JPH3 increases the level of cytosolic Ca²⁺ and calpain activation in colorectal and gastric cancer cells.

JPH3 suppressed pro-survival Bcl2 family members and Akt phosphorylation

Most stimuli that induce mitochondria-dependent apoptosis promote the permeabilization of the outer mitochondrial membrane, which is controlled by proteins of the Bcl2 family. So we checked pro-survival members Bcl2, p-Bcl2 (Ser70), p-Bcl2 (Thr56), Mcl-1, Bcl-xL and the pro-apoptotic proteins Bid, Bad, Bax, and Bak by Western blot. JPH3 expression suppressed Bcl2 and p-Bcl2 (Ser70) and the other pro-survival members Mcl-1 and Bcl-xL but did not affect the pro-apoptotic proteins (Figure 5D). We also checked some important protein kinases which play a critical role in controlling cell survival and mitochondrial apoptosis. JPH3 expression only significantly suppressed Akt phosphorylation (p-Akt at S473) (Figure 5E). These data showed that JPH3 induces mitochondrial apoptosis through suppressing pro-survival Bcl2 family members and Akt phosphorylation.

Correlation of JPH3 expression with clinicopathological features of digestive cancer patients

To evaluate JPH3 expression and methylation in digestive primary tumors, we analyzed *JPH3* methylation by MSP in 53 colorectal and 32 gastric tumor samples with paired normal tissues. *JPH3* methylation was higher in tumors than paired normal tissues (Figure 6A). BGS confirmed the MSP results in primary tumors (Figure 6B). Moreover, *JPH3* methylation is significantly correlated with its low expression (Figure 6E, Table 3).

JPH3 protein expression was next investigated by immunohistochemistry in normal and cancer tissues, including 10 normal colonic and 10 normal gastric mucosa biopsy samples, 137 colorectal and 192 gastric cancer paraffin blocks. Representative immunohistochemical staining results are shown in Figure 6C. JPH3 was found to be highly expressed in the cytoplasm of epithelial and some stromal cells in normal colonic and gastric mucosa. In contrast, JPH3 expression was absent in most primary tumor samples. 56.9% (78/137) of colorectal and 39.6% (76/192) of gastric tumors showed weak or moderate expression, relative to its expression in normal mucosa.

The correlation between clinicopathological parameters of colorectal and gastric cancer patients and JPH3 protein expression are summarized in Table 1 and Table 2. In colorectal cancer, low JPH3 protein expression was significantly correlated with high T, N and Duke stages ($p=0.023$, 0.007 and 0.023 respectively). In gastric cancer, low JPH3 protein expression was significantly correlated with poor differentiation ($p=0.000$), high clinical stage ($p=0.018$), more lymph node metastases ($p=0.008$), more liver metastases ($p=0.025$) and invasion ($p=0.019$). More importantly, the difference of 5-year overall survival rates for colorectal and gastric patients between JPH3 high and low expression were both statistically significant (Figure 6D).

In addition, we also retrieved *JPH3* methylation and expression data of digestive tumors in Cancer Genome Atlas (TCGA) database (Figure 6F). *JPH3* expression levels in colorectal ($n=379$) and gastric ($n=415$) tumors are lower than that in normal controls, while *JPH3* methylation levels were significantly higher in colorectal ($n=233$) and gastric ($n=48$) tumors than normal controls. Moreover in colorectal cancer, *JPH3* expression and methylation have significant correlation with clinical stages ($\rho=0.2898852$, $p=2.322e-08$) including T stages ($\rho=0.2548168$, $p=5.709e-07$) and N stages ($\rho=0.251135$, $p=8.387e-07$). These results suggest that *JPH3* downregulation by promoter CpG methylation is correlated with disease progression and poor survival of colorectal and gastric cancer patients.

Table 1. Association between JPH3 protein expression and clinicopathological parameters in 137 primary colorectal tumor cases

Clinicopathological parameters	n	JPH3 protein expression		P-value
		Low expression n=87 (63.5%)	High expression n=50 (36.5%)	
Sex				
Male	79	46 (58.2%)	33 (41.8%)	
Female	58	41 (70.7%)	17 (29.3%)	0.134
Age				
<63	70	46 (65.7%)	24 (34.3%)	
≥63	67	41 (61.2%)	26 (38.8%)	0.583
Differentiation				
Well	72	41 (56.9%)	31 (43.1%)	
Moderate	46	31(67.4%)	15 (32.6%)	
Poor	19	15 (78.9%)	4 (21.1%)	0.166
pT categories				
T1/T2	31	15 (48.4%)	16 (51.6%)	
T3/T4	106	72 (67.9%)	34 (32.1%)	0.047
pN categories				
pN0	69	36 (52.2%)	33 (47.8%)	
pN1/2	68	51 (75%)	17 (25%)	0.006
pM categories				
pM0	122	74 (60.7%)	48 (39.3%)	
pM1	15	13 (86.7%)	2 (13.3%)	0.048
Duke stage				
A/B	66	34 (51.5%)	32 (48.5%)	
C/D	71	53 (74.6%)	18 (25.4%)	0.005

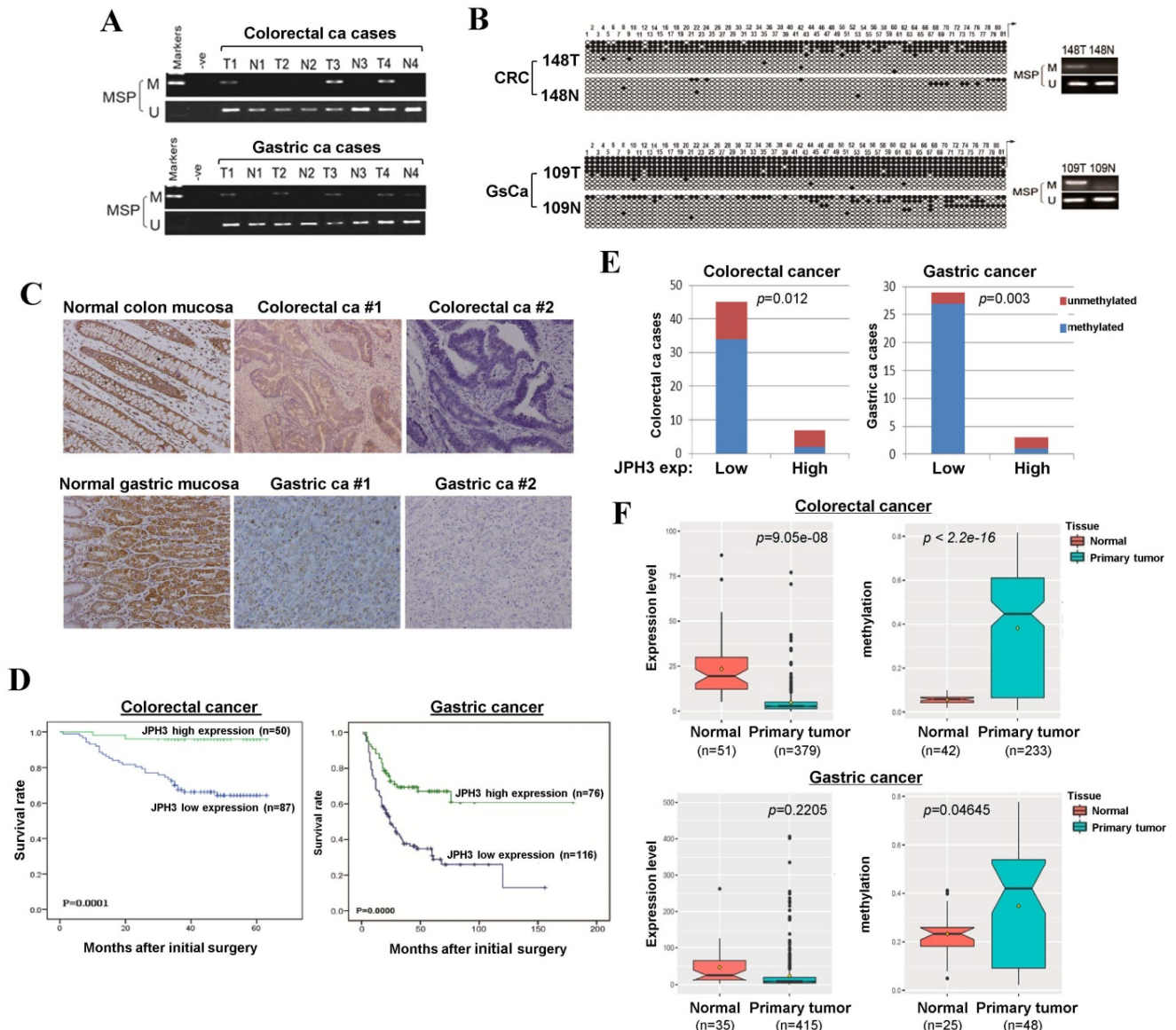


Figure 6. JPH3 reduction by promoter methylation in colorectal and gastric primary tumors and its correlation with clinicopathological features. Representative analysis of *JPH3* methylation in primary tumors (T) and normal tissues (N) by (A) MSP and (B) BGS. (C) Representative immunohistochemical staining for JPH3 in normal gastric and colonic mucosa, as well as their primary carcinoma tissues. Original magnification: 200x. (D) Overall survival analysis results in colorectal and gastric cancer patients. (E) Correlation of *JPH3* methylation and expression in primary colorectal and gastric tumors. (F) Analysis of *JPH3* expression and methylation in colorectal and gastric cancer patients from the TCGA database, as well as their correlation with clinicopathological parameters.

Discussion

JPHs stabilize the junctional membrane complexes (JMC) between PM and ER, which are the structure for cell surface and ion channel cross-talking [20, 21]. JPH proteins contain eight N-terminal “membrane occupation and recognition nexus” (MORN) domains, a space-spanning alpha helix, and a C-terminal “transmembrane domain” (TMD). The MORN domains mediate binding to the plasma membrane, while the hydrophobic TMD is anchored into the SR membrane [14]. JPHs are found to be highly conserved in evolution through analysis of

over 60 JPH proteins from over 40 species, in particular the MORN motifs found in all species [22].

JPHs family contains four members, JPH1-4. JPH1 is predominantly expressed in skeletal muscle; JPH2 is distributed in both skeletal muscle and heart; JPH3 and JPH4 are abundantly expressed in brain. JPH1 or JPH2 knock-out in mice induces disorganized JMC and disrupted Ca²⁺ homeostasis in skeletal muscle, and finally neonatal or embryonic death, respectively [23, 24]. JPH1 and JPH2 knockdown study in mouse skeletal muscle fibers also showed decreases in intracellular Ca²⁺ release and the disorganization of JMC [25]. In the case of the

neuronal JPH isoforms (JPH3 and JPH4), JPH3 null mice exhibit adult onset, progressive motor dysfunction, whereas JPH3 hemizygous mice have a similar but milder phenotype. The only information that has been reported about JPH3 involves a mouse model for neurodegenerative HDL2 disorder that showed a decrease in JPH3 expression due to changes at the genomic level [26, 27]. Loss of JPH3 protein expression contributes to HDL2 pathogenesis perhaps through disruption of calcium flux or ER dysregulation [17]. No information is available so far for the possible roles of JPHs during human carcinogenesis yet.

Table 2. Association between JPH3 protein expression and clinicopathological parameters in 192 primary gastric tumor cases

Clinicopathological parameters	n	JPH3 protein expression		P-value
		Low expression n=116 (60.4%)	High expression n=76 (39.6%)	
Sex				
Male	138	83 (60.1%)	55 (39.9%)	0.946
Female	54	33 (61.1%)	21 (38.9%)	
Age				
>62	95	57 (60%)	38 (40%)	0.907
≤62	97	59 (60.8%)	38 (39.2%)	
Differentiation				
Well and Moderate	75	33 (44%)	42 (56%)	0.000
Poor	117	83 (70.9%)	34 (29.1%)	
Clinical stage				
I	43	21 (48.8%)	22 (51.2%)	0.018
II	40	18 (45%)	22 (55%)	
III	76	52 (68.4%)	24 (31.6%)	
IV	33	25 (75.8%)	8 (24.2%)	
Lymph node metastasis				
Absent	86	43 (50%)	43 (50%)	0.008
Present	106	73 (68.9%)	33 (31.1%)	
Liver metastasis				
Absent	157	89 (56.7%)	68 (43.3%)	0.025
Present	35	27 (77.1%)	8 (22.9%)	
Invasion				
T1-2	62	30 (48.4%)	32 (51.6%)	0.019
T3-4	130	86 (66.2%)	44 (33.8%)	

Table 3. Correlation between JPH3 expression and methylation in colorectal and gastric cancers

	JPH3 promoter	No.	JPH3 protein expression		P-value
			Low expression	High expression	
CRC	M	36	34	2	0.012
	U	16	11	5	
GsCa	M	28	27	1	0.003
	U	4	2	2	

JPH3 has 4 splicing variants, with variant 1 as the full-length transcript encoding a fully functional 748-amino acid protein; while variant 2 and 3 are much shorter isoforms and variant 4 is a non-coding RNA. We found that JPH3 variant 1,2,4 were

frequently downregulated or silenced in CRC and gastric tumor cell lines and tumors, while variant 3 is never expressed. Thus in this study, we mainly studied JPH3 variant 1 in digestive system tumorigenesis. Whether other JPH3 variants also play some roles in tumorigenesis need further investigation. We also checked the expression of a related member JPH4 in tumor cell lines and primary cases (Suppl. Figure S1). JPH4 was frequently downregulated in tumor cells and primary tumors, while no correlation of JPH4 and JPH3 expression was detected. The functions of JPH4 in cancer pathogenesis need further investigations.

DNMT1 and DNMT3B are two major DNA methyltransferases responsible for the maintenance and *de novo* CpG methylation, and their disruption results in >95% loss of overall genomic methylation as well as CpG island demethylation [28]. The colon cell line pair (HCT116 and DNMT1/DNMT3B double knock-out (DKO)) provides us with a useful model system to identify key methylated cancer genes involved in colon tumorigenesis, which has been widely used by other researchers [18, 29, 30]. Through methylome analysis of HCT116 and DKO pair cell line, we identified JPH3 as a methylated TSG candidate in colon cancer. We found that in addition to its abundant expression in neuronal cells, JPH3 is also readily expressed in some other normal tissues including digestive systems. For the first time, we report that JPH3 was frequently silenced or reduced in digestive tumors due to its promoter CpG methylation, indicating its potential value as a biomarker for these cancers in future. As clinical primary tumor tissue samples always contain infiltrating non-malignant cells, it is well known that unmethylated TSG promoter alleles are always detected in primary tumor tissue samples, which actually acts as a good positive control for the integrity of bisulfited tumor genomic DNA. Thus, we also successfully detected unmethylated JPH3 alleles in all the primary tumor tissue samples (Figure 6).

Meanwhile, restoring JPH3 expression inhibits tumor cell growth *in vitro* and *in vivo*, indicating that JPH3 acts as a tumor suppressor in colorectal and gastric cancers. We also demonstrated that its tumor suppressor function is, at least partially, due to its induction of mitochondrial-mediated apoptosis through unfolded protein response involving perturbed calcium homeostasis. Previous studies show that JPHs are essential for the maintenance of JMC structures and thus participates in the maintenance of intracellular Ca²⁺ homeostasis and regulation of intracellular calcium signaling pathways [14]. We also found that JPH3 expression increased the cytosolic Ca²⁺ level to about twice that of the

controls. So as in neuronal cells, JPH3 expression could also participate in the maintenance of intracellular Ca^{2+} homeostasis in colorectal and gastric tumor cells.

Endoplasmic reticulum serves two major functions in the cells. The newly synthesized proteins destined for secretion, cell surface or intracellular organelles are properly folded in this organelle and ER also provides the cells with a Ca^{2+} reservoir and controls the free and bound Ca^{2+} levels in the cells [31-33]. Though cancer cells always reprogramme their metabolism to support the rapid proliferation, eventually loss of nutrients/energy leads to accumulation of unfolded and misfolded proteins in ER and disruption of Ca^{2+} homeostasis. So tumor cells often suffer from more ER stress than do normal cells. Since ER is also an important sensor for cellular stress, a group of signal transduction pathways will be activated to maintain ER homeostasis upon ER stress in human cancers. Two important pathways are the unfolded protein response (UPR) and autophagy [34, 35].

We found that in colorectal and gastric cancer cells, JPH3 expression significantly induced UPR but not autophagy. The initial purpose of UPR is to stop the unpropor protein translation and reestablish homeostasis and normal ER function and meanwhile increase production of molecular chaperones involved in correct protein folding [36, 37]. However, when the ER stress is extensive or sustained, the

adaptive mechanisms fail to reestablish homeostasis and the normal function of ER, the UPR pathways may launch apoptotic pathways to remove the stressed cells [38]. This is consistent with our results that JPH3 expression significantly induced apoptosis after ER stress induction, but did not significantly alter spontaneous or 5-fluorouracil induced apoptosis. Additionally, JPH3 expression significantly elevated the expression level of CHOP and TRB3 which both function to mediate programmed cell death.

In addition to activation of UPR, JPH3 expression also induce calpain activation and subsequent mitochondrial membrane depolarization, followed by mitochondrial release of Cyto C, Smac and consequent activation of caspase -9 and -3. Moreover, JPH3 expression increased PARP cleavage and induced AIF nuclear translocation. Both pro- and anti-apoptotic Bcl2 family members play an important role in the regulation of outer mitochondrial membrane permeability [39]. However, our results indicated that JPH3 expression mainly suppresses Bcl2 and p-Bcl2 (Ser70) and the pro-survival members Mcl-1 and Bcl-xL, but does not affect pro-apoptotic proteins. As 16q24 LOH is frequently present in multiple other solid tumors, 16q24 is also a TSG locus for common tumors other than digestive cancers. Whether epigenetic disruption of *JPH3* is involved in the pathogenesis of other solid tumors needs further investigation.

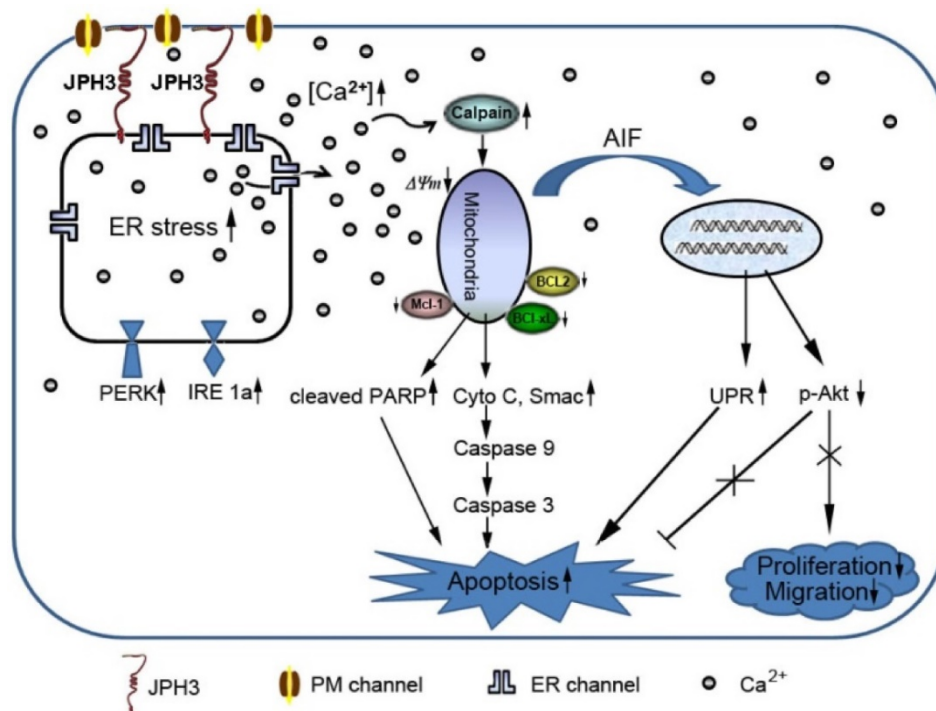


Figure 7. Diagram showing the mechanisms with which JPH3 expression induced cancer cell apoptosis by unfolded protein response involving perturbed calcium homeostasis.

In conclusion, JPH3 was found to be a novel tumor suppressor downregulated and methylated in colorectal and gastric cancers, which increases mitochondrial-mediated apoptosis through UPR involving perturbed calcium homeostasis (Figure 7). This appears to be the first report that a junctional complex junctophilin family protein is involved in tumor pathogenesis. JPH3 may potentially serve as a biomarker colorectal and gastric cancer. Further studies on the detailed intracellular Ca²⁺ signaling pathways affected by JPH3 expression could lead to a better understanding of the complex regulation of calcium homeostasis in normal and cancer cells, as well as identify new therapeutic strategies for gastrointestinal cancer.

Materials and Methods

Cell lines, tumor samples, and normal tissues

A panel of gastric (KatoIII, YCC1, YCC3, YCC6, YCC7, YCC9, YCC11, SNU719, AGS, MKN-45, SNU1, SNU16, MKN-28) and colon cancer cell lines (SW480, SW620, DLD1, Colo320, HT-29, LoVo, RKO, HCT116, HCT116-DKO with double knock-out of *DNMT1* and *DNMT3B*) were used. Cell lines were maintained in RPMI or DMEM medium (Gibco BRL, Rockville, MD) with 10% fetal bovine serum. Human normal adult tissue RNA samples were purchased commercially (Stratagene, La Jolla, CA, or Millipore Chemicon, Billerica, MA).

A total of 192 gastric cancer patients and 137 colorectal cancer patients who underwent surgery between Feb 2004 and June 2006 at the Sir Run Run Shaw Hospital (Hangzhou, Zhejiang, China) were investigated. Patients who received pre-operative chemotherapy were excluded. 10 normal gastric and 10 normal colon mucosa biopsy samples were used as normal controls. This study was approved and monitored by the ethics committee of Sir Run Run Shaw Hospital, Zhejiang University.

Establishment of paired colon cancer cell line methylomes by MeDIP-chip

Methylated DNA immunoprecipitation (MeDIP) coupled with promoter microarray hybridization (MeDIP-chip) was performed as previously [19]. Briefly, methylated HCT116 and DKO cell DNA was immunoprecipitated by monoclonal antibody against 5-methylcytidine (33D3, Diagenode, Seraing, Belgium), and then hybridized to NimbleGen™ HG18 Meth (385K CGI plus) promoter arrays (Array Star, Inc., MD). Bioinformatics analysis of methylome data was performed as previously [19].

5-aza-2'-deoxycytidine (5-Aza) and trichostatin A (TSA) treatment

Cells with silenced JPH3 expression were seeded at a density of 1×10⁶ cells/ml. After overnight culture, cells were treated with the demethylation agent 5-aza-2'-deoxycytidine (5-Aza; Sigma-Aldrich, St Louis, MO) at a final concentration of 10 μM for 72 hours and then further treated with 100 nM trichostatin A (TSA; Cayman Chemical Co, Ann Arbor, MI) for 16 hours. Cells were subsequently harvested for DNA and RNA extraction.

Bisulfite treatment and promoter methylation analysis

Bisulfite modification of DNA, methylation specific PCR (MSP) and bisulfite genome sequencing (BGS) were carried out as described previously [40, 41]. MSP and BGS primers are listed in Suppl. Table S1. Genomic positions of MSP and BGS primers are shown in Suppl. Figure S2.

JPH3-expressing plasmid and cell transfection

The *JPH3*-expressing plasmid was kindly provided by Prof. Keith Robertson (Mayo Clinic, Rochester, MN) and sequence verified. Colon cell line HCT116 and gastric cell line MKN-28 were transfected with pCMV-Tag2A-JPH3 plasmid (Agilent, Santa Clara, California) or empty vector as a control using MegaTran 1.0 transfection reagent (Origene, Maryland, MD). Stable JPH3-expressing clones were selected for further studies.

Determination of mitochondrial transmembrane potential

The cells were trypsinized, washed in ice-cold phosphate buffered saline, and stained with JC-1 (5μg/ml; Molecular Probes) for 30 min at 37°C in 5% CO₂ atmosphere. Then, the changes in the mitochondrial transmembrane potential (MMP) were monitored and analyzed. The percentage of red and green fluorescence was estimated by flow cytometry.

Measurement of intracellular Ca²⁺

Change in the intracellular Ca²⁺ level was analyzed using Fluo-3 AM, a cell permeant, Ca²⁺-sensitive fluorescent dye. Cells were loaded with 2μM Fluo-3 AM diluted in Krebs-Ringer buffer [KRB; 0.7mM Na₂HPO₄, 1.5mM NaH₂PO₄ (pH 7.4), 10mM d-glucose, 120mM NaCl, 4.5mM KCl, 0.5mM MgCl₂] for 30min in a CO₂ incubator. After washing with KRB to remove the residual dye, the cells were harvested, washed with Ca²⁺-free PBS and analyzed by flow cytometry.

Calpain activity assay

Calpain activity was measured using the cell-permeable fluorogenic calpain substrate, S-LLVY-AMC. In brief, cells were harvested, washed twice with HEPES-buffered Hank's balanced salt solution (HBSS; pH 7.4) without phenol red, resuspended in HEPES-HBSS at 3×10^5 cells/ml, and prewarmed at 37°C in a CO₂ incubator for 10 min. The substrate S-LLVY-AMC (25 μM) was then added, and the AMC fluorescence intensity was measured using a spectrofluorometer (BIO-TEK ELX800) at excitation and emission wavelengths of 400 and 500 nm, respectively.

Subcellular fractionation

Nuclear and cytoplasmic protein extractions were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Biotech, Peking, China) and mitochondrial fractions were isolated using the Cell Mitochondria Isolation Kit (Beyotime Biotech, Peking, China).

In vivo subcutaneous tumor model

All *in vivo* experimental protocols were approved by the animal care committee of Sir Run Run Shaw Hospital, Zhejiang University. Viable MKN-28 cells (3.5×10^6 cells in 0.1 ml phosphate buffer saline) were injected subcutaneously into the right dorsal flank of 6-week-old female BALB/c nude mice (six mice per group). Tumor volume was assessed every 2 days for 4 weeks. Tumor volume was calculated using the following formula: (short diameter)² × (long diameter) / 2.

TCGA database analysis

JPH3 expression and methylation of were analyzed using The Cancer Genome Atlas (TCGA) database. *JPH3* expression and processed clinical data of gastric and colorectal patients in TCGA were obtained from Fire browse database (<http://www.firebrowse.org>). For colorectal cancer, a total of 379 tumor samples and 51 normal samples were studied. For gastric cancer, a total of 415 tumor samples and 35 normal samples were studied. Gene expression and methylation levels were measured using normalized log₂ RSEM index from FireBrowse database. Wilcoxon rank sum test was used to analyze the significance in differential gene expression. Pearson method was applied as a correction to multiple comparisons.

Statistical analysis

The results in the figures are all expressed as mean values ± s.d. Statistical analysis was performed in SPSS 11.0 for Windows (SPSS Inc., Chicago, IL,

U.S.). The two-tailed Chi-square test was used to analyze the association of *JPH3* protein expression with the different clinicopathological parameters. For all tests, $p < 0.05$ was considered to be of statistical significance.

Abbreviations

TSG, tumor suppressor gene; LOH, Loss of heterozygosity; HD, Huntington's disease; HDL2, Huntington's disease-like 2; JPH, Junctophilin; JPH3, Junctophilin 3; JMC, junctional membrane complex; 5-Aza, 5-aza-2'-deoxycytidine; TSA, trichostatin A; MSP, methylation-specific PCR; BGS, bisulfite genome sequencing; HBSS, Hank's balanced salt solution; CGI, CpG island; BFA, brefeldin A; EMT, epithelial-mesenchymal transition; MMP, mitochondrial transmembrane potential; UPR, unfolded protein response; PM, plasma membrane; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; MORN, membrane occupation and recognition nexus; TMD, transmembrane domain; HDL2, Huntington disease-like 2.

Supplementary Material

Supplementary figures and tables.

<http://www.thno.org/v07p2150s1.pdf>

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Competing Interests

The authors have declared that no competing interest exists.

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