

Characterization of DAPK1 as a novel transcriptional target of BRMS1

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Abstract. Breast cancer metastasis suppressor 1 (BRMS1) can specifically regulate tumor metastasis in many cancers. Our previous studies have demonstrated that BRMS1 can promote cell apoptosis through regulating osteopontin (OPN) expression in hepatocellular carcinoma (HCC) cells. However, the transcriptional targets of BRMS1 have not been thoroughly studied. In this study, death-associated protein kinase 1 (*DAPK1*), a tumor suppressor gene with multiple roles in regulating cell death, was identified as a potential transcriptional target of BRMS1 in the whole genome expression microarray. Quantitative real-time PCR and western blot analysis of HCC cells overexpressing BRMS1 further confirmed the transcriptional regulation relationship between BRMS1 and *DAPK1*. Moreover, *DAPK1* expression was frequently decreased or even lost in HCC tissue samples by comparison with neighboring pathologically normal liver tissue, which was consistent with the decreased BRMS1 expression pattern. To unravel the molecular mechanism of BRMS1 in regulating *DAPK1*, a series of deletion mutants of *DAPK1* promoter was subjected to luciferase assay. The luciferase units of -200 to -80 bp region, with two tandem putative NF- κ B binding sites, were specifically enhanced by BRMS1 expression. Site-directed mutants of NF- κ B binding sites blocked the transcriptional activation effect. In addition, the binding capability of BRMS1 and the putative NF- κ B binding

sites were demonstrated in the chromatin immunoprecipitation (ChIP) assay. In conclusion, our study characterized *DAPK1* as a novel transcriptional target of BRMS1. Transcriptional activation of *DAPK1* might be another important mechanism accounting for the metastasis suppressive activity of BRMS1.

Introduction

Hepatocellular carcinoma (HCC) is among the most common and highly lethal cancers worldwide, with a depressingly low long-term survival rate (1). So far, tumor metastasis remains the primary cause of death for most HCC patients. Tumor metastasis consists of several discrete biological processes, initiating from escaping primary tumor site, invading and surviving in the surrounding tissues, entering the lymphatic vessels or the bloodstream, eventually transporting to a remote site and forming new colonies (2).

Breast cancer metastasis suppressor 1 (*BRMS1*) is an active tumor metastasis suppressor gene, exhibiting tumor metastasis suppressive activity in breast cancer, melanoma and non-small cell lung cancer (3-5). Many studies have reported that the expression of *BRMS1* was silenced due to gene mutation or promoter hypermethylation in metastatic tumor cells (6-8). Functional studies revealed that *BRMS1* was involved in the regulation of cell-cell communication, cell migration, cell invasion, cell apoptosis and tumor angiogenesis, while no obvious effect has been shown on cell proliferation, cell-matrix adhesion and matrix degradation (9). Mechanistically, *BRMS1* is an essential part of mSin3a-HDAC complex, which modulates gene transcription activity by regulating the acetylation levels of both histone and transcriptional factors (10). Therefore, several cancer-related genes have been recently characterized as transcriptional targets of *BRMS1*, including *CXCR4*, *cLAP-2*, *Bcl-xL*, *uPA* and *miR146* (9). We have previously demonstrated that *BRMS1* was able to sensitize HCC cells to apoptosis through suppressing NF- κ B signaling pathway and osteopontin expression (11,12).

In this study, we started from gene expression microarray and identified a novel *BRMS1* target gene, death-associated protein kinase 1 (*DAPK1*), in HCC cells. *DAPK1* is a well-defined tumor suppressor gene, with significant suppressive effect in both tumor growth and metastasis *in vivo* (13). *DAPK1*

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participates in multiple cell death-related signaling pathways, including caspase-dependent cell apoptosis, mitochondrial-dependent cell apoptosis and autophagic cell death (14). In a variety of tumor tissues and cell lines, the promoter region of *DAPK1* was hypermethylated, resulting in significant decrease or loss of *DAPK1* expression (15,16). Additionally, many transcriptional factors including TP53, C/EBP- β , HSF1 and SMAD can transcriptionally activate *DAPK1* expression, while STAT3 and NF- κ B play the opposite role (17).

We reported for the first time that BRMS1 could transcriptionally activate *DAPK1* expression in HCC cells. Immunohistochemical analysis of human HCC tissues revealed that *DAPK1* expression was specifically silenced in tumor cells. The association relationship between *BRMS1* and *DAPK1* expression in paired HCC tissues was studied through western blot analysis and the transcriptional mechanism of BRMS1 on *DAPK1* promoter was further elucidated. Our findings suggested a functional relationship between BRMS1 and *DAPK1*, indicating another potential molecular mechanism accounting for BRMS1's tumor suppressive role in HCC cells.

Materials and methods

Ethics statement. This study was accomplished with the approval of the Medical Ethics Committee of School of Life Sciences, Fudan University, Shanghai, China.

Tumor specimens. Fresh surgical specimens of HCC, including tumor tissues and the neighboring pathologically non-tumorous liver tissues, were obtained from liver cancer patients at Zhongshan Hospital (Fudan University, Shanghai, China). All of the samples were immediately frozen in liquid nitrogen after surgery and then stored at -80°C before further analysis.

Tissue microarray analysis. Fifty matched pairs of tumor samples and adjacent normal tissues from clinical HCC patients were used for the construction of a tissue microarray (Shanghai Biochip Co., Ltd. Shanghai, China) as previously described (18). In brief, sections (4 μ m thickness, 2 mm diameter) were taken from individual paraffin-embedded tissues and precisely arrayed on 3-aminopropyltriethoxysilane-coated slides for subsequent staining with an anti-DAPK1 antibody (Sigma, USA). The immunohistochemistry analysis was performed in Shanghai Biochip. All the images were visualized by Leica DC 500 camera on a microscope equipped with Leica DMRA2 fluorescent optics (LEICA).

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Life Technologies, USA), and 1-2 μ g of RNA was used for reverse transcription using PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan). PCR analysis was performed using the SYBR Green Supermix kit (Takara) with the CFX Connection detection system (Bio-Rad, USA). Diluted cDNA was used in a 10- μ l real-time PCR reaction in triplicate for each gene and each sample. Cycle parameters were 95°C for 5 min hot start and 40 cycles of 95°C for 5 sec, 58°C for 10 sec and 72°C for 20 sec. Blank controls with no cDNA templates were performed to rule out contamination. The specificity of the PCR product was confirmed by melting curve analysis.

BRMS1 primers were: forward, 5'-ACTGAGTCAGCTGCGTTGCGG-3'; reverse, 5'-AAGACCTGGAGCTGCCTCTGGCGTGC-3'. *DAPK1* primers were: forward, 5'-TGTCTTCCACCAACTCCAGCAG-3'; reverse, 5'-AAATCGCCAATCCATTCAAATAAGC-3'. 18S *rRNA* primers were: forward, 5'-GTAACCCGTTGAACCCCAT-3'; reverse, 5'-CCATCCAATCGGTAGTAGCG-3'.

The expression levels of all genes were normalized to those of the house keeping gene 18S *rRNA*. Relative gene expression levels were calculated by the formula $2^{-\Delta Ct}$, where ΔCt (Critical threshold) = Ct of genes of interest - Ct of 18S *rRNA*.

Plasmid construction. Full length of *DAPK1* promoter was amplified from human genomic DNA, forward, 5'-CACTCACTCCCTAGCTGTGT-3'; reverse, 5'-TAGCCCCCTCATGCA-3'. The amplicons were separated by DNA electrophoresis and purified before being cloned into the pGEM-T Easy Vector (Promega, USA). Both the full-length promoter and three 3' deletion mutants were further cloned into pGL3-Basic Vectors (Promega). The relevant primers were as follows: pGL3-Basic-*DAPK1*-P(P): forward, 5'-GGGGTACCCACTCACTCCCTAGCTGTGT-3'; reverse, 5'-CCAAGCTTTAGCCCCCTCATGCA-3'. pGL3-Basic-*DAPK1*-P1(P1): forward, 5'-GGGGTACCCACTCACTCCCTAGCTGTGT-3'; reverse, 5'-CCAAGCTTGACCGGGTCTCCGGA-3'. pGL3-Basic-*DAPK1*-P2(P2): forward, 5'-GGGGTACCCACTCACTCCCTAGCTGTGT-3'; reverse, 5'-CCAAGCTTCCACCTCCAGGACG-3'. pGL3-Basic-*DAPK1*-P3(P3): forward, 5'-GGGGTACCCACTCACTCCCTAGCTGTGT-3'; reverse, 5'-CCAAGCTTGCGACTCCCTCTCC-3'. Two site-directed mutations of pGL3-Basic-*DAPK1*-P, Mut1 and Mut2, were obtained by KOD-Plus-Mutagenesis kit (Toyobo, Japan) according to the manufacturer's instructions. The relevant primers were as follows: Mut1: forward, 5'-TCTGAGCGCCGGGGAGGTCTACTTCCTTTT-3'; reverse, 5'-AACCGCTCGCTGAAGACCGGTCTCCGGAG-3'. Mut2: forward, 5'-AGGGATACTTCCTTTTGGGGTTGCCATTTT-3'; reverse, 5'-CAACGGCGCTAGACCCCGCTCGCTGAAGA-3'. Recombinant pCMV-Myc-BRMS1 were constructed as previously described (11).

Cell culture and transfection. Human embryonic kidney cell line 293T and HCC cell line SK-Hep1 were all cultured with Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C in 5% CO₂ humidified atmosphere. Cells at 80% confluency were transfected using Lipofectin 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Dual-luciferase assay. Cells were seeded into 24-well culture plates at a density of 1×10^5 /well and transfected with indicated *DAPK1* promoter constructs or pGL3-Basic empty vectors. The pRL-TK control vector (20 ng/well) was used for normalization. Cells were harvested 36 h after transfection. Firefly and *Renilla* activities were determined using GloMax 96 Microplate Luminometer (Promega) according to the manufacturer's instructions. Data are presented as the changes in Firefly luciferase activity relative to *Renilla* luciferase activity.

Western blot analysis. Protein samples were separated by 10% SDS-PAGE gel and then transferred to PVDF membranes.

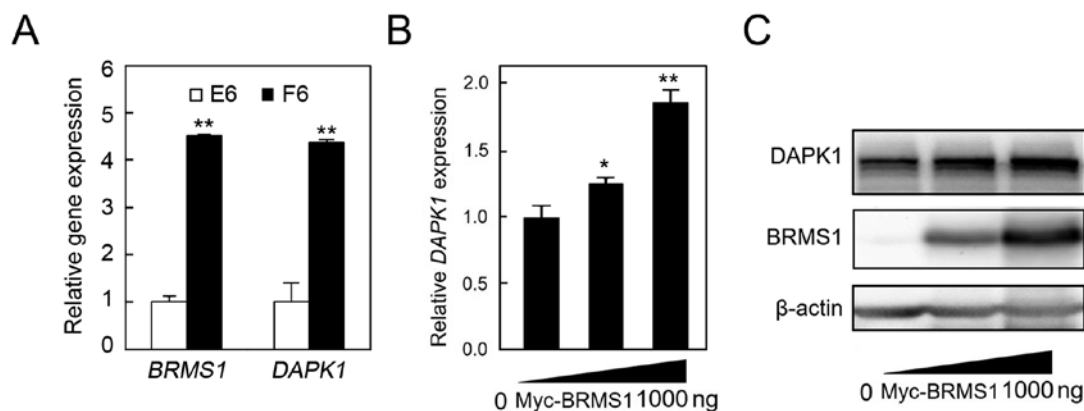


Figure 1. Human *DAPK1* was transcriptionally activated by BRMS1. (A) Relative *BRMS1* and *DAPK1* mRNA expression levels in SK-Hep1 cell clone stably expressing *BRMS1* (F6) and control cell clone (E6) were analyzed through gene expression microarray. Values are normalized to internal control and expressed as mean \pm SD, n=3. (B) Relative *DAPK1* expression levels in SK-Hep1 cells transiently expressing different dosages of pCMV-Myc-BRMS1 (Myc-BRMS1) were quantified through qRT-PCR. Values are normalized to internal control 18S *rRNA* and expressed as mean \pm SD, n=3. (C) Western blot analysis of DAPK1 and BRMS1 protein expression levels in SK-Hep1 cells transiently expressing different dosages of Myc-BRMS1. β -actin was used as a loading control.

Non-specific binding was blocked by incubation with 5% fat-free milk for 1 h at room temperature. After blocking, the membranes were incubated with specific primary antibodies against different proteins at 4°C overnight, followed by incubation with HRP-conjugated secondary antibody for 45 min at room temperature. Immunoreactivity was visualized by enhanced chemiluminescence (Pierce, USA) on a molecular imager ChemiDoc XRS⁺ system (Bio-Rad). Related antibodies included the mouse monoclonal antibody against BRMS1 (Abcam, USA), β -actin and Myc tag (Sigma), the rabbit monoclonal antibody against DAPK1 (Sigma), peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Jackson, USA).

Chromatin immunoprecipitation (ChIP). The ChIP assay was performed according to Farenham laboratory protocol (19). Basically, HEK293T cells were seeded at 1×10^7 /dish in 100-mm dishes and transfected with pCMV-Myc-BRMS1 plasmid or empty pCMV-Myc vector. At 36 h post-transfection, cells were subject to crosslinking and fixation using 4% formaldehyde (Amresco, USA). Cell nuclei were released by SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1) before sonication. Fragmented genomic DNA was immuno-precipitated by anti-Myc antibody at 4°C after precleared with protein A-agarose beads. Mouse IgG was used as the negative control to exclude genomic contamination. The immune-precipitated DNA fragments were then collected, washed, de-crosslinked and digested with Proteinase K. DNA fragments were recovered by phenol/chloroform extraction and amplified through qRT-PCR using the following primers: primers covering the putative NF- κ B binding site (P-A): forward, 5'-CAGCGAGCGGGTCTTAG-3'; reverse, 5'-GTA AAATGGCAACCCCAAAA-3'. Primers covering the downstream region of the putative NF- κ B binding site (P-B): forward, 5'-TCTTCAAAAGGACTGGAGACTGA-3'; reverse, 5'-CCTGCCAAGTTCCTCGCC-3'.

Statistical analysis. Comparisons of quantitative data were analyzed by Student's t-test. Categorical data were analyzed

by Fisher's exact test. We considered $p < 0.05$ to be different and $p < 0.01$ to be significant different.

Results

DAPK1 is positively regulated by BRMS1 in HCC cells. In order to find novel transcriptional targets of BRMS1, BRMS1 stably expressing clone F6 and control clone E6 established in our previous study were analyzed in a whole genome expression microarray (11). Under the selection criteria of |Fold change| >2, 260 potential target genes were found, of which 194 genes were upregulated and the other 66 genes were suppressed by BRMS1 expression (data not shown). A well-studied tumor suppressor gene, *DAPK1* was found to be 4.46-fold overexpressed in cells stably expressing BRMS1 by comparison with control cells (Fig. 1A). Next, qRT-PCR and western blot analyses were carried out to confirm this result. Different dosages of recombinant BRMS1 plasmid were transiently introduced into SK-Hep1 cells. As shown in Fig. 1B and C, both the mRNA and the protein expression levels of *DAPK1* were, not surprisingly, upregulated upon BRMS1 overexpression in a dose-dependent manner. The data strongly suggest that *DAPK1* is a potential transcriptional target of BRMS1 in HCC cells.

DAPK1 is remarkably downregulated in human HCC tissues. To assess the expressional pattern of *DAPK1* in HCC tissues, immunohistochemical analysis was carried out on a tissue microarray including 50 paired HCC tissues and adjacent non-tumorous liver tissues. It was revealed that the immunostaining signal of *DAPK1* is reduced in 37 tumor tissues by comparison with adjacent non-tumorous tissues, of which, 18 tumor tissues exhibited remarkably suppressed or even silenced *DAPK1* expression. Images from four representative HCC samples composed of both tumor cells and normal liver cells are shown in Fig. 2A. In contrast to high *DAPK1* expression in normal liver cells, *DAPK1* immunostaining signal is totally lost in the surrounding tumor cells. To further confirm the expression pattern of *DAPK1* in HCC samples, additional

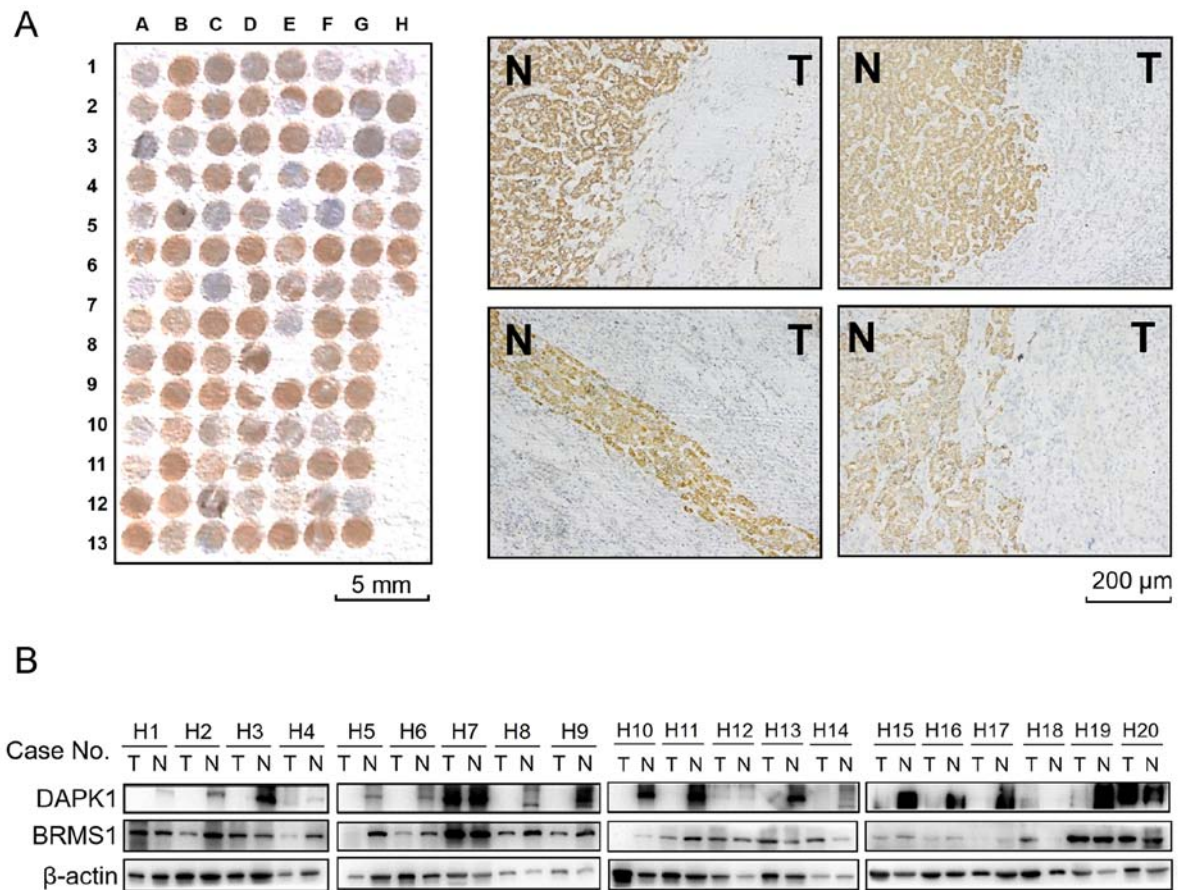


Figure 2. DAPK1 is remarkably downregulated in paired HCC specimens. (A) A tissue microarray composed of 50 paired HCC specimens with corresponding non-tumorous tissues was subjected to immunohistochemical staining using the anti-DAPK1 antibody. Left panel, the full image of the tissue microarray. Right panel, representative images derived from four HCC specimens with both tumor cells (T) and normal liver cells (N). (B) Western blot analysis of DAPK1 and BRMS1 protein expression in other 20 paired (case no., H1-H20) HCC specimens (T) with corresponding non-tumorous tissues (N). β -actin was used as a loading control.

20 paired HCC tissues and adjacent non-tumorous liver tissues were subjected to western blot analysis (Fig. 2B). Consistently, DAPK1 protein expression was almost completely lost in 15 out of 20 tumor tissues. We have previously investigated the BRMS1 expression in these HCC samples (11), whether DAPK1 expression was associated with BRMS1 expression in these paired protein samples needed to be assessed. It was shown that in all 11 samples with downregulated BRMS1 expression, 10 samples exhibited consistent DAPK1 downregulation (positive ratio = 90.91%). Moreover, only 5 out of the other 9 samples without suppressed BRMS1 expression exhibited silenced DAPK1 (positive ratio = 55.56%). This finding indicates a potential correlation of endogenous BRMS1 and DAPK1 expression in clinical HCC tissues, providing another piece of evidence that DAPK1 might be transcriptionally regulated by BRMS1.

DAPK1 promoter is transcriptionally activated by BRMS1 in a dose-dependent manner. To clarify the transcriptional mechanism between BRMS1 and *DAPK1*, the promoter region (-1,084 to -80 bp) of human *DAPK1* gene was cloned from human genomic DNA and a series of 3' deletion mutants, namely P1 (-1,084 to -200 bp), P2 (-1,084 to -436 bp) and P3 (-1,084 to -627 bp), were constructed subsequently (Fig. 3A). The four DNA fragments were then inserted into pGL3-Basic vector

to detect their transcriptional activity in the luciferase assay, respectively. As shown in Fig. 3B, by comparison with full length promoter, both pGL3-Basic-P1(P1) and pGL3-Basic-P2(P2) truncates exhibited a 50% reduction in the report gene's activity, and pGL3-Basic-P3(P3) almost lost the entire transcriptional activity. This result suggests that -200 to -80 bp and -627 to -436 bp are two potential transcriptional active regions in *DAPK1* promoter. Next, Myc-BRMS1 plasmid was co-introduced into cells to investigate the transcriptional effect of BRMS1 on *DAPK1* promoter. It was found that BRMS1 can transcriptionally activate the luciferase activity of pGL3-Basic-P(P), whereas no effect was observed on the other three truncates (Fig. 3C). Moreover, when different dosages of Myc-BRMS1 plasmid were utilized, it was shown that the luciferase activity of pGL3-Basic-P gradually increased upon the expression of exogenous BRMS1 (Fig. 3D). Taken together, current results indicate that BRMS1 might be able to activate *DAPK1* expression through the -200 to -80 bp region of the promoter.

BRMS1 is able to bind the putative NF- κ B binding sites of DAPK1 promoter. After identifying -200 to -80 bp region of *DAPK1* promoter as the target of interest, we screened this region using online transcriptional element prediction software to uncover the potential transcriptional factor binding

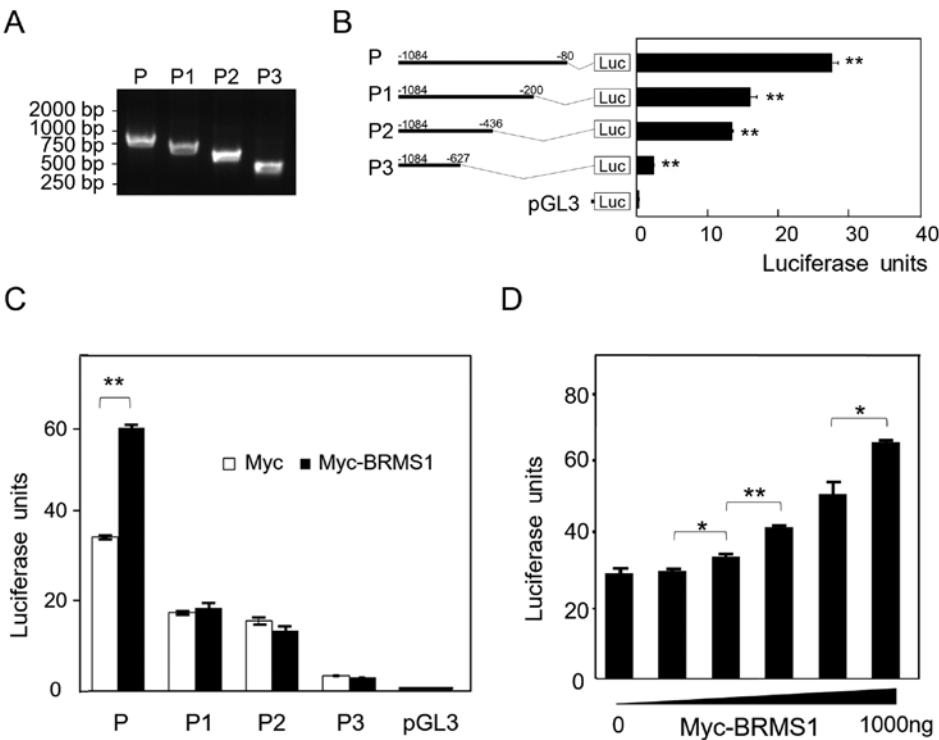


Figure 3. BRMS1 is able to transcriptionally activate *DAPK1* promoter's activity. (A) Gel electrophoresis analysis of the full length *DAPK1* promoter (P) and three deletion mutants (P1, P2 and P3). (B) Schematic of four recombinant luciferase reporter constructs (left panel) and relative reporter activities in HEK293T cells (right panel). Values are expressed as mean \pm SD, n=3. (C) Recombinant Myc-BRMS1 or empty vector was transfected into HEK293T cells together with indicated recombinant luciferase reporter constructs. Reporter activity values are expressed as mean \pm SD, n=3. (D) Relative reporter activities were analyzed in cells co-transfected with pGL3-P and different dosages of Myc-BRMS1. Reporter activity values are expressed as mean \pm SD, n=3. *P<0.05; **P<0.01.

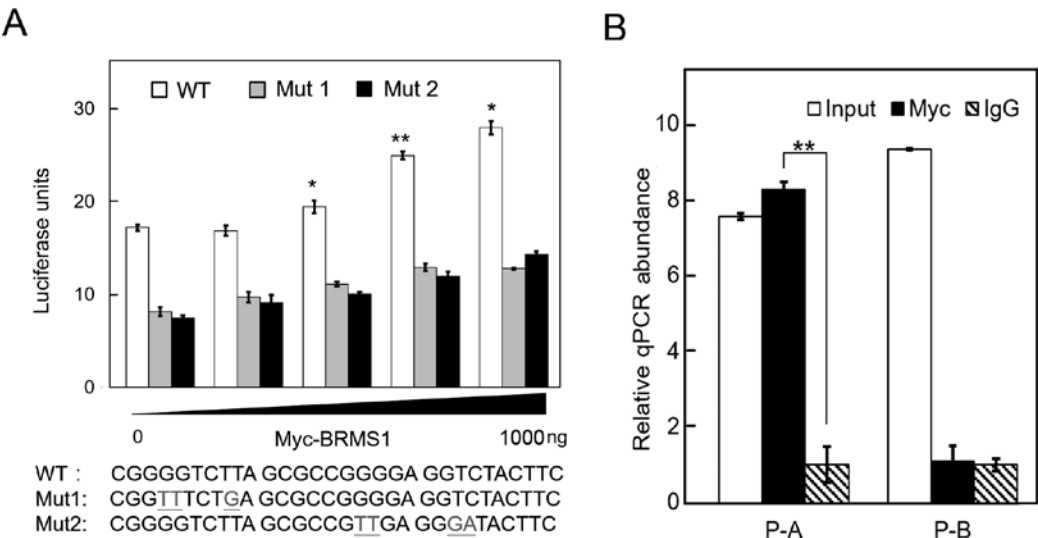


Figure 4. Exogenous BRMS1 binds to the putative NF- κ B binding site of *DAPK1* promoter. (A) Nucleotide sequences of the putative NF- κ B binding sites of wild-type *DAPK1* promoter (WT) and two site-directed mutants (Mut1 and Mut2) are shown in the bottom. Mutant nucleotides are underlined and indicated in grey. Relative reporter activities were analyzed in cells co-transfected with indicated reporter constructs and Myc-BRMS1. Values are expressed as mean \pm SD, n=3. (B) ChIP analysis of the putative NF- κ B binding site in *DAPK1* promoter. Soluble chromatin was prepared from HEK293T cells transfected with Myc-BRMS1 and immunoprecipitated by an anti-Myc antibody or IgG control. Two different pairs of primers (P-A, P-B) were utilized in the qRT-PCR analysis. Blank controls with no DNA templates were utilized to rule out contamination and soluble chromatin before immunoprecipitation was utilized as input control. Values are expressed as mean \pm SD, n=3. *P<0.05; **P<0.01.

sites. The -190 to -181 bp (CGGGGTCTTA) region and the -176 to -167 bp (CGGGGAGGTC) region of *DAPK1* promoter were predicted to be two tandem putative binding sites for transcription factor NF- κ B, which is demonstrated to be an important deacetylation modification target of BRMS1 (20-22). To assess whether BRMS1 functions through these binding sites, two site-directed point-mutations of pGL3-Basic-P, Mut1 and Mut2, were constructed. As shown in Fig. 4A, both

mutations markedly abolished the transcriptional activation effect of BRMS1 on *DAPK1* promoter, strongly suggesting that these putative NF- κ B binding sites might be involved in BRMS1-*DAPK1* transcriptional regulation. Chromatin immunoprecipitation experiment was further carried out in order to demonstrate whether BRMS1 could bind to this *DAPK1* promoter region. Cells overexpressing Myc-BRMS1 were lysed and immunoprecipitated by anti-Myc antibody or IgG control. Two different pairs of primers were designed to target the two putative NF- κ B binding sites (P-A) and the downstream region of NF- κ B binding sites (P-B), respectively. As shown in Fig. 4B, only the promoter region containing NF- κ B binding sites was immunoprecipitated by specific antibody against exogenous BRMS1. These data together demonstrate that BRMS1 is able to bind *DAPK1* promoter through the putative NF- κ B binding sites.

Discussion

After BRMS1 was demonstrated to be a functional partner of mSin3a-HDAC complex involved in regulating chromatin status and gene expression, several BRMS1-target genes have been demonstrated to be involved in BRMS1-mediated tumor metastasis suppression. Herein, we reported for the first time that *DAPK1* was another transcriptional target of BRMS1 in HCC cells. *DAPK1* was initially identified as a positive mediator of apoptosis activated by interferon- γ in HeLa cell (23). Soon after that, *DAPK1* was well demonstrated as an important tumor suppressor, which also functions in suppressing tumor metastasis (24). For example, Inbal *et al* found that restoration of *DAPK1* to physiological levels in high-metastatic Lewis carcinoma cells successfully suppressed tumor metastasis in a mouse model (25). By utilizing *in situ* TUNEL staining of tumor sections, they proposed that loss of *DAPK1* expression provides a unique mechanism that links suppression of apoptosis to metastasis. Kuo *et al* reported that *DAPK1* can block cell migration and invasion in tumor cells by blocking the integrin-mediated polarity pathway (26). Additionally, Chen *et al* revealed that miR-103/107 promote metastasis of colorectal cancer probably through targeting *DAPK1* (27). It is therefore of great interest to uncover the relationship between BRMS1 and *DAPK1* in HCC tissues and cell lines.

Consistent with the tumor suppressive function of *DAPK1*, clinical studies also revealed that the expression of *DAPK1* is significantly decreased in chronic lymphocytic leukemia, breast cancer and head and neck cancer (16,28,29). Importantly, loss of *DAPK1* expression is associated with advanced tumor stages and tumor metastasis (24). Studies of *DAPK1* expression pattern and regulation mechanism in HCC are limited. Matsumoto *et al* investigated the expression of *DAPK1* in 43 Japanese HCC patients (30). Through association study, they found *DAPK1*-negative HCC cases were associated with high serum AFP, lower tumor differentiation, and less apoptosis. However, while they revealed that the status of *DAPK1* protein expression correlated with IFN- γ -receptor and Fas expression, but not the promoter methylation status, how IFN- γ -receptor and Fas control *DAPK1* expression in HCC was not addressed.

In our study, by utilizing 70 pairs of Chinese HCC specimens, *DAPK1* was found to be remarkably reduced or even lost in HCC tissues by comparison with neighboring non-

tumorous tissues, which is consistent with Matsumoto *et al* (30). Moreover, the transcriptional regulation mechanism of *DAPK1* has been carefully studied through luciferase assay and ChIP experiment. Two tandem NF- κ B binding sites locating in -190 to -181 bp and -176 to -167 bp of *DAPK1* promoter could be recognized by BRMS1, and responsible for BRMS1-mediated transcriptional activation. It has been noted that NF- κ B is an important modification substrate of BRMS1-mSin3a-HDAC complex (10). BRMS1 suppresses several metastasis-related genes through deacetylating NF- κ B subunits (21,22,31). However, Shanmugam *et al* recently reported that NF- κ B was able to bind to *DAPK1* promoter together with HDACs and played a negative role in regulating *DAPK1* expression in acute myeloid leukemia (32). More interestingly, Li *et al* found that the cell cycle regulator ING4 was specifically induced by BRMS1 through suppressing NF- κ B activities as well, because NF- κ B functions as a transcriptional inhibitor of ING4 (33). Based on all these pieces of evidence, we speculate that BRMS1 might also be able to upregulate *DAPK1* expression through releasing *DAPK1* promoter from the negative regulation of NF- κ B. Further experiments are in progress to demonstrate this hypothesis and elucidate the underlying molecular mechanism. It would also be important and interesting to investigate how BRMS1 and *DAPK1* collaborate to regulate tumor metastasis.

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References

1. Tang Z-Y, Ye S-L, Liu Y-K, Qin LX, Sun HC, Ye QH, Wang L, Zhou J, Qiu SJ, Li Y, *et al*: A decade's studies on metastasis of hepatocellular carcinoma. *J Cancer Res Clin Oncol* 130: 187-196, 2004.
2. Fidler IJ: The pathogenesis of cancer metastasis: The 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 3: 453-458, 2003.
3. Seraj MJ, Samant RS, Verderame MF and Welch DR: Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. *Cancer Res* 60: 2764-2769, 2000.
4. Shevde LA, Samant RS, Goldberg SF, Sikaneta T, Alessandrini A, Donahue HJ, Mauger DT and Welch DR: Suppression of human melanoma metastasis by the metastasis suppressor gene, BRMS1. *Exp Cell Res* 273: 229-239, 2002.
5. Smith PW, Liu Y, Siefert SA, Moskaluk CA, Petroni GR and Jones DR: Breast cancer metastasis suppressor 1 (BRMS1) suppresses metastasis and correlates with improved patient survival in non-small cell lung cancer. *Cancer Lett* 276: 196-203, 2009.
6. Nagji AS, Liu Y, Stelow EB, Stukenborg GJ and Jones DR: BRMS1 transcriptional repression correlates with CpG island methylation and advanced pathological stage in non-small cell lung cancer. *J Pathol* 221: 229-237, 2010.
7. Li J, Cheng Y, Tai D, Martinka M, Welch DR and Li G: Prognostic significance of BRMS1 expression in human melanoma and its role in tumor angiogenesis. *Oncogene* 30: 896-906, 2011.
8. Liu Y, Mayo MW, Nagji AS, Smith PW, Ramsey CS, Li D and Jones DR: Phosphorylation of RelA/p65 promotes DNMT-1 recruitment to chromatin and represses transcription of the tumor metastasis suppressor gene BRMS1. *Oncogene* 31: 1143-1154, 2012.

9. Kodura MA and Souchelnytskyi S: Breast carcinoma metastasis suppressor gene 1 (BRMS1): Update on its role as the suppressor of cancer metastases. *Cancer Metastasis Rev* 34: 611-618, 2015.
10. Meehan WJ, Samant RS, Hopper JE, Carrozza MJ, Shevde LA, Workman JL, Eckert KA, Verderame MF and Welch DR: Breast cancer metastasis suppressor 1 (BRMS1) forms complexes with retinoblastoma-binding protein 1 (RBP1) and the mSin3 histone deacetylase complex and represses transcription. *J Biol Chem* 279: 1562-1569, 2004.
11. Wu Y, Jiang W, Wang Y, Wu J, Saiyin H, Qiao X, Mei X, Guo B, Fang X, Zhang L, *et al*: Breast cancer metastasis suppressor 1 regulates hepatocellular carcinoma cell apoptosis via suppressing osteopontin expression. *PLoS One* 7: e42976, 2012.
12. Wu J, Wang Y, Qiao X, Saiyin H, Zhao S, Qiao S and Wu Y: Cloning and characterization of a novel human BRMS1 transcript variant in hepatocellular carcinoma cells. *Cancer Lett* 337: 266-275, 2013.
13. Bialik S and Kimchi A: The death-associated protein kinases: Structure, function, and beyond. *Annu Rev Biochem* 75: 189-210, 2006.
14. Bialik S and Kimchi A: The DAP-kinase interactome. *Apoptosis* 19: 316-328, 2014.
15. Yegnasubramanian S, Kowalski J, Gonzalgo ML, Zahurak M, Piantadosi S, Walsh PC, Bova GS, De Marzo AM, Isaacs WB and Nelson WG: Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Cancer Res* 64: 1975-1986, 2004.
16. Raval A, Tanner SM, Byrd JC, Angerman EB, Perko JD, Chen SS, Hackanson B, Grever MR, Lucas DM, Matkovic JJ, *et al*: Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. *Cell* 129: 879-890, 2007.
17. Benderska N and Schneider-Stock R: Transcription control of DAPK. *Apoptosis* 19: 298-305, 2014.
18. Gao Q, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, Xu Y, Li YW and Tang ZY: Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol* 25: 2586-2593, 2007.
19. Boyd KE, Wells J, Gutman J, Bartley SM and Farnham PJ: c-Myc target gene specificity is determined by a post-DNA-binding mechanism. *Proc Natl Acad Sci USA* 95: 13887-13892, 1998.
20. Cicek M, Fukuyama R, Cicek MS, Sizemore S, Welch DR, Sizemore N and Casey G: BRMS1 contributes to the negative regulation of uPA gene expression through recruitment of HDAC1 to the NF-kappaB binding site of the uPA promoter. *Clin Exp Metastasis* 26: 229-237, 2009.
21. Cicek M, Fukuyama R, Welch DR, Sizemore N and Casey G: Breast cancer metastasis suppressor 1 inhibits gene expression by targeting nuclear factor-kappaB activity. *Cancer Res* 65: 3586-3595, 2005.
22. Samant RS, Clark DW, Fillmore RA, Cicek M, Metge BJ, Chandramouli KH, Chambers AF, Casey G, Welch DR and Shevde LA: Breast cancer metastasis suppressor 1 (BRMS1) inhibits osteopontin transcription by abrogating NF-kappaB activation. *Mol Cancer* 6: 6, 2007.
23. Deiss LP, Feinstein E, Berissi H, Cohen O and Kimchi A: Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev* 9: 15-30, 1995.
24. Chen HY, Lee YR and Chen RH: The functions and regulations of DAPK in cancer metastasis. *Apoptosis* 19: 364-370, 2014.
25. Inbal B, Cohen O, Polak-Charcon S, Kopolovic J, Vadai E, Eisenbach L and Kimchi A: DAP kinase links the control of apoptosis to metastasis. *Nature* 390: 180-184, 1997.
26. Kuo JC, Wang WJ, Yao CC, Wu PR and Chen RH: The tumor suppressor DAPK inhibits cell motility by blocking the integrin-mediated polarity pathway. *J Cell Biol* 172: 619-631, 2006.
27. Chen HY, Lin YM, Chung HC, Lang YD, Lin CJ, Huang J, Wang WC, Lin FM, Chen Z, Huang HD, *et al*: miR-103/107 promote metastasis of colorectal cancer by targeting the metastasis suppressors DAPK and KLF4. *Cancer Res* 72: 3631-3641, 2012.
28. Levy D, Plu-Bureau G, Decroix Y, Hugol D, Rostène W, Kimchi A and Gompel A: Death-associated protein kinase loss of expression is a new marker for breast cancer prognosis. *Clin Cancer Res* 10: 3124-3130, 2004.
29. Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, Jen J, Herman JG and Sidransky D: Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Res* 60: 892-895, 2000.
30. Matsumoto H, Nagao M, Ogawa S, Kanehiro H, Hisanaga M, Ko S, Ikeda N, Fujii H, Koyama F, Mukogawa T, *et al*: Prognostic significance of death-associated protein-kinase expression in hepatocellular carcinomas. *Anticancer Res* 23B: 1333-1341, 2003.
31. Liu Y, Mayo MW, Xiao A, Hall EH, Amin EB, Kadota K, Adusumilli PS and Jones DR: Loss of BRMS1 promotes a mesenchymal phenotype through NF-kB-dependent regulation of Twist1. *Mol Cell Biol* 35: 303-317, 2015.
32. Shanmugam R, Gade P, Wilson-Weekes A, Sayar H, Suvannasankha A, Goswami C, Li L, Gupta S, Cardoso AA, Al Baghdadi T, *et al*: A noncanonical Flt3ITD/NF-kappaB signaling pathway represses DAPK1 in acute myeloid leukemia. *Clin Cancer Res* 18: 360-369, 2012.
33. Li J and Li G: Cell cycle regulator ING4 is a suppressor of melanoma angiogenesis that is regulated by the metastasis suppressor BRMS1. *Cancer Res* 70: 10445-10453, 2010.