AMPK activators suppress breast cancer cell growth by inhibiting DVL3-facilitated Wnt/β-catenin signaling pathway activity

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Abstract. Adenosine monophosphate-activated protein kinase (AMPK) is a principal regulator of metabolism and the conservation of energy in cells, and protects them from exposure to various stressors. AMPK activators may exhibit therapeutic potential as suppressors of cell growth; however, the molecular mechanism underlying this phenomenon in various cancer cells remains to be fully elucidated. The present study investigated the effects of AMPK activators on breast cancer cell growth and specified the underlying molecular mechanism. In the present study, the AMPK activator metformin impaired breast cancer cell growth by reducing dishevelled segment polarity protein 3 (DVL3) and β-catenin levels. Western blotting and immunohistochemistry demonstrated that DVL3 was recurrently upregulated in breast cancer cells that were not treated with metformin, and was significantly associated with enhanced levels of β-catenin, c-Myc and cyclin D1. Overexpression of DVL3 resulted in upregulation of β-catenin and amplification of breast cancer cell growth, which confirmed that Wnt/β-catenin activation via DVL3 is associated with breast cancer oncogenesis. To elucidate the underlying mechanism of these effects, the present study verified that metformin resulted in a downregulation of DVL3 and β-catenin in a dose-dependent manner, and induced phosphorylation of AMPK. Compound C is an AMPK inhibitor, which when administered alongside metformin, significantly abolished the effects of metformin on the reduction of DVL3 and activation of the phosphorylation of AMPK. Notably, the effects of metformin on the mRNA expression levels of DVL3 remain to be fully elucidated; however, a possible interaction with DVL3 at the post-transcriptional level was observed. It has previously been suggested that the molecular mechanism underlying AMPK activator-induced suppression of breast cancer cell growth involves an interaction with, and impairment of, DVL3 proteins. The results of the present study are of future clinical importance and advocate the use of metformin as a potential therapeutic agent against breast cancer.

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Introduction

Breast cancer is a highly prevalent type of cancer, which is associated with a high mortality rate and affects women worldwide (1). Genetic, metabolic and lifestyle-associated risk factors may be important in the onset and progression of breast cancer. Obesity is a risk factor that contributes to the initiation and progression of breast cancer, via increased circulation of estrogen, insulin, insulin-like growth factor and adipokines (1). Mutations in oncogenes and growth regulatory genes are further variable factors that may be associated with the development of the disease. Aberrant activation of the Wnt/β-catenin signaling pathway, resulting from accumulation of β-catenin, has been observed to be critically associated with carcinogenesis, including breast cancer (2-4). Potentially targeting the Wnt/β-catenin signaling pathway may provide a novel molecular approach to cancer therapy.

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a key regulator of the cellular energy system (5). It regulates cellular metabolism and protects living cells from the environmental stressors they may be exposed to, including hypoxia and nutrient deficiency, which lead to elevations in cellular AMP:adenosine triphosphate ratio (6). Structurally, mammalian AMPK is a heterotrimeric complex composed of one catalytic α subunit (63 kDa) and two regulatory subunits each, of β and γ (38 and 36 kDa, respectively); each of which has multiple isoforms (α1 and α2; β1 and β2; γ1, γ2 and γ3). AMP binds to the γ subunit of AMPK and induces its allosteric activation (5,6). AMPK activation impairs the mammalian target of rapamycin (mTOR) signaling pathway, and its confirmatory p70S6 kinase and 4E-BP1 activity (7), inhibiting protein synthesis and cell growth. Notably, low AMPK activity favors carcinogenesis (8,9). Various AMPK activators have been investigated for their antitumor effects in several types of human cancer, including A23187, A769662, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR)

and metformin (10-13). Metformin in particular has been used in several clinical trials (13-15). The underlying molecular mechanisms by which these agents exhibit their antitumor effects remain to be elucidated. AMPK may prevent tumorigenesis via various mechanisms (16), including activation of an upstream kinase, liver kinase B1 (LKB1). LKB1 is a proven tumor suppressor that coordinates cell polarity in part via AMPK signaling, which leads to disorganized cell division due to cross-talk with the pro-proliferative Wnt signaling pathway and growth-restrictive AMPK-mTOR metabolic pathway, in various types of carcinoma, including gastrointestinal, pancreatic, ovarian and breast cancer (17).

The present study investigated the interaction of AMPK with the Wnt/β-catenin signaling pathway and its associated components in breast carcinogenesis. Dishevelled segment polarity protein (DVL) 3 was observed to be significantly upregulated and correlated with Wnt/β-catenin activity in breast cancer cell growth. Furthermore, AMPK activators exhibited the potential to inhibit breast cancer cell growth by diminishing the DVL3-mediated upregulation of Wnt/β-catenin signaling. The activation of AMPK led to DVL3-mediated Wnt/β-catenin reduction; however, this was significantly abrogated by an AMPK inhibitor. The results of the present study verified the importance of the role of DVL3 in breast cancer tumorigenesis and emphasized the potential therapeutic value of targeting DVL3 via AMPK activators in the treatment of breast cancer.

Materials and methods

Cell culture and treatments. MCF-7, MDA-MB-231 and T-47D breast cancer cell lines, and the MCF-10 healthy breast cell line were procured from the American Type Culture Collection (Manassas, VA, USA). MCF-7 and MDA-MB-231 cells were grown in Minimum Essential Media (MEM), T-47D cells were grown in RPMI-1640 media and MCF-10 cells were grown in Mammary Epithelial Cell Growth Medium (Lonza, Basel, Switzerland). MEME and RPMI-1640 were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Media were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and cells were cultured in the presence of 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere containing 5% CO_2. AMPK activators AICAR (Sigma-Aldrich, Merck Millipore, Darmstadt, Germany) and A23187 (Calbiochem, EMD Millipore, Billerica, MA, USA) were used to treat MCF-7 cells. AICAR was used at 0, 1 and 2 mM and A23187 at 0, 2 and 4 mM for 24 h followed by protein isolation and western blotting. Another AMPK activator, metformin (Sigma-Aldrich, Merck Millipore) was used to treat MCF-7 cells at 0, 20 and 40 mM concentrations for 24 h followed by subsequent analyses. An AMPK inhibitor, Compound C (Sigma-Aldrich, Merck Millipore) was used to pre-treat MCF-7 cells at 5 mM for 2 h followed by subsequent treatments and analyses.

Plasmids and cell transfection. For expression of green fluorescent protein (GFP)/DVL3 protein, a GFP-tagged-DVL3 expressing construct was generated by amplifying DVL3 using primer 1 (5'-GTGCTGGAATTCCCGAGGCC-3') and primer 2 (5'-GCTCACATTGGATCCACAAAG-3'), with pEGFP-C1 plasmid (Addgene, Cambridge, MA, USA) serving as the negative control. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for MCF-7 and MDA-MB-231 cell transfection according to the manufacturer's protocol. Transfected cells were selected with antibiotic G418 (Sigma-Aldrich, Merck Millipore) for 2 weeks. Western blotting was performed to verify the cells that stably expressed GFP-DVL3.

Total RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to synthesize cDNA according to the manufacturer's protocol. The expression of DVL genes was assessed by RT-qPCR using the ABI PRISM™ 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with Taqman Gene Expression Assays, as per manufacturer's protocol. The TaqMan gene expression assays contained prevalidated primers and TaqMan probes for the individual genes specifically custom synthesized by Applied Biosystems. For amplification of DVL genes, two sets of primers and Taqman probes were used. The probes were labeled at the 5' end with the reporter molecule 6-carboxy-fluorescein and labeled at the 3' end with the quencher molecule 6-carboxy-tetramethyl-rhodamine. Primer sequences were: DVL2: 5'-TGAGCAACG ATGACGCTGTG-3' (forward) and 5'-GCAGGGTCAATT GGCTGGA-3' (reverse); DVL3: 5'-ACAATGCCAAGCTAC CATGCTTC-3' (forward) and 3'-AGCTCCGATGGGTTA TCAGCAC-5' (reverse); and DVL-1: 5'-CCTTCCATCCAA ATGTTGC-3' (forward) and 5'-GTGACTGACCATAGACTC TGTGC-3' (reverse). The RT-PCR amplification conditions were set at: 95˚C for 5 min, 95˚C for 1 min, 58˚C for 30 sec, and 72˚C for 1 min, for 40 cycles. Relative quantification of gene expression was performed as described by Applied Biosystems using 18S rRNA as an internal control. The comparative CT (cycle threshold) method was used for relative quantification of DVL genes' mRNA (18). PCR was performed in accordance with the manufacturer's protocol, in triplicate, on an ABI 7500 real-time PCR system (Applied Biosystems).

Protein isolation and western blotting. Cells were prepared as per experimental requirement, and harvested and pelleted for protein isolation. Cell pellets were lysed with 1X lysis buffer composed of 20 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1% sodium vanadate, 1 mM PMSF together with protease inhibitor cocktail (1:100; including phenylmethane sulfonyl fluoride). The cell lysates were centrifuged at 12, 000 x g for 20 min at 4˚C, supernatant was collected and total protein content was estimated by using BCA protein assay kit (Sigma-Aldrich, Merck Millipore). For western blot analysis, 20 μg protein for each sample was separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. These membranes were then subsequently blocked with 5% skimmed milk (fat-free) for 30 min and incubated with specific primary antibodies overnight at 4˚C. The primary antibodies used were procured from Cell Signaling Technology, Inc. (Danvers, MA, USA),

Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and EMD Millipore. Anti-phosphorylated (p) AMPKα (catalog no. cs 2531; 1:1,000), anti-AMPKα (catalog no. cs 2532; 1:1,000), anti-c-Myc (catalog no. cs 5605; 1:1,000), anti-cyclin D1 (catalog no. cs 2978; 1:1,000), anti-DVL1 (catalog no. sc-8025; 1:1,000), anti-DVL2 (catalog no. sc-8026; 1:1,000), anti-DVL3 (catalog no. sc-8027; 1:1,000), anti-β-catenin (catalog no. sc-7963; 1:1,000), and anti-GAPDH antibody (catalog no. EMD AB2302; 1:2,000) as a loading control. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary anti-mouse (catalog no. cs 7076) or anti-rabbit antibodies (catalog no. cs 7074), obtained from Cell Signaling Technology, Inc. in accordance with the manufacturer's protocol. An enhanced chemiluminescence solution (Invitrogen; Thermo Fisher Scientific, Inc.) was used for signal detection using photographic film followed by membrane scanning.

Immunohistochemistry (IHC). IHC was performed in healthy and cancerous breast tissue (n=5/each). Cancerous breast tissue samples and their corresponding healthy tissue samples were procured at the time of surgical resection, from patients presenting at the Department of Breast Surgery, The Third Hospital of Nanchang, Jiangxi, China. The study was approved by the ethics committee of The First Affiliated Hospital of Nanchang University, (Nanchang, China; Reference no. 2123432524AK) and written informed consent was obtained from patients or their family. Patients included in the study were Chinese adult women, mean age, 40.2±5.67. The patients had never received chemotherapy prior to surgical resection. For IHC analysis, surgically resected tissue parts were fixed in normal buffered formalin followed by serial dehydration in graded alcohol then embedded in paraffin. Tissues embedded in paraffin blocks were cut into 4 μM thick sections. Tissue sections were de-paraffinized and treated with primary antibodies for anti-DVL3 (1:200 dilution) and anti-β-catenin (1:200 dilution) for 24 h at 4˚C, while Tris-buffered saline served as a negative control. Antibodies were procured from Novus Biologicals, LLC (Littleton, CO, USA) and BD Biosciences (Franklin Lakes, NJ, USA), respectively. Sections were visualized under a light microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 40x magnification and manual scoring of the expression levels of proteins was calculated from immunopositive staining area (0-100%). The intensity of staining was recorded on the scale of: +1, weak; +2, moderate; +3, intense; and +4, very intense. The fold-change in the expression level of each protein was calculated by normalization to the expression level of the same protein in the healthy breast section. The IHC analysis from each tissue section was examined and scored independently by two investigators. The breast tumor array (BC08022) analysis for DVL3 and β-catenin was performed by US Biomax, Inc. (Rockville, MD, USA). The expression levels of DVL3 and β-catenin were represented as fold-change of each gene as compared to normal tissue. The cutoff point (6-folds) of both genes was determined by its expression levels and statistical significance.

Cell viability assay. The XTT cell proliferation kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to measure cell viability. The assay was performed according to the manufacturer's protocol. Each cell line was assayed in triplicate and three independent experiments were performed.

Clonogenic assay. Cells were grown and seeded for 24 h. Media was removed after 24 h, followed by incubation with methanol for 30 min. Cells were then stained with crystal violet for 1 h at room temperature, and subsequently washed with PBS followed by cell counting under a light microscope (Leica Microsystems GmbH) at 40x magnification using Image J software version 1.4 (U.S. National Institutes of Health, Bethesda, USA). The experiment was performed in triplicate and three independent experiments were performed.

Statistical analysis. Data were analyzed using one-way analysis of variance followed by Tukey's post hoc test, and the Chi-square test. Data are presented as the mean ± standard deviation. SPSS v16 was used for statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DVL3 is recurrently upregulated in breast cancer cells. It has previously been demonstrated that DVLs are key in the upregulation of the expression of β-catenin and promotion of cell growth in various cancers, such as colorectal cancer (19), malignant pleural mesothelioma (20) and non-small-cell lung cancer (21). The present study first examined the expression patterns of DVL1, 2 and 3, in MCF-7, MDA-MB-231 and T-47D breast cancer cell lines, and the MCF-10 healthy breast cell line by western blot analysis. The results demonstrated that DVL3 was notably upregulated in breast cancer cell lines, compared with the healthy breast cell line (Fig. 1A). These data suggested that DVL3 is upregulated in breast cancer cells. The MCF-7 cells revealed greater levels of DVL3 compared with the other breast cancer cell lines. The mRNA expression levels of DVL genes in breast cancer cell lines and the healthy breast cell line were then determined by RT-qPCR. The gene expression analysis demonstrated similar results to those presented in Fig. 1A. The mRNA expression levels of DVL3 were considerably greater in breast cancer cell lines compared with healthy breast cells (Fig. 1B). Furthermore, the mRNA expression levels of DVL1 and DVL2 were reduced in each cell line, compared with DVL3. These findings primarily indicated that DVL3 was the major isoform among DVLs that was recurrently upregulated in breast cancer cells. The results also suggested that MCF-7 and MDA-MB-231 cell lines were more susceptible to alterations in DVL3 levels, compared with T-47D cells. MCF-7 and MDA-MB-231 cell lines were therefore selected for further studies.

DVL3 and β-catenin are overexpressed in breast cancer. The significance of DVL3 and β-catenin expression level alterations in breast cancer was investigated by examining the expression levels of these proteins using IHC and tissue array analysis in breast cancer and healthy breast tissues. The IHC analysis of breast cancer and healthy breast tissues revealed a greater level of staining of DVL3 and β-catenin in breast cancer tissues compared with healthy breast tissue (Fig. 2A). The relative protein expression levels of DVL3 and

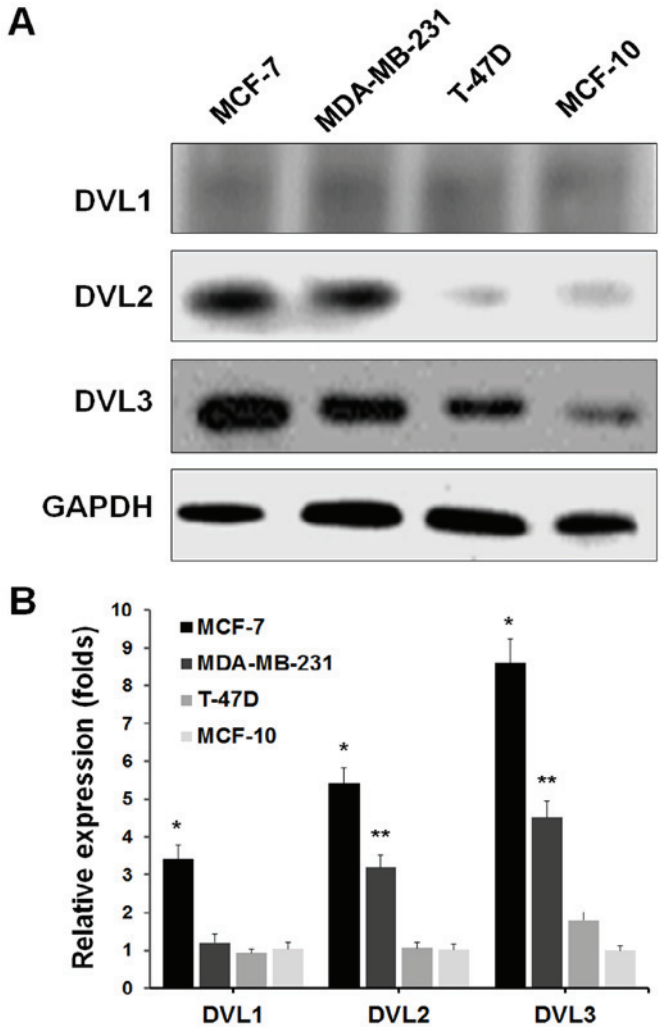

Figure 1. DVL3 upregulation in breast cancer cell lines. (A) Western blot analysis of the expression of DVL1, 2 and 3 in MCF-7, MDA-MB-231 and T-47D breast cancer cells, and the MCF-10 healthy breast cell line. GAPDH served as a loading control. (B) Reverse transcription-quantitative polymerase chain reaction analysis of human breast cancer and healthy breast cell lines, to quantify mRNA expression levels of DVL1, 2 and 3. DVL-1, *P=0.003 vs. MCF-10; DVL-2, *P=0.002 vs. MCF-10, **P=0.004 vs. MCF-10; DVL-3, *P=0.0006 vs. MCF-10, **P=0.002 vs. MCF-10. DVL, dishevelled segment polarity protein.

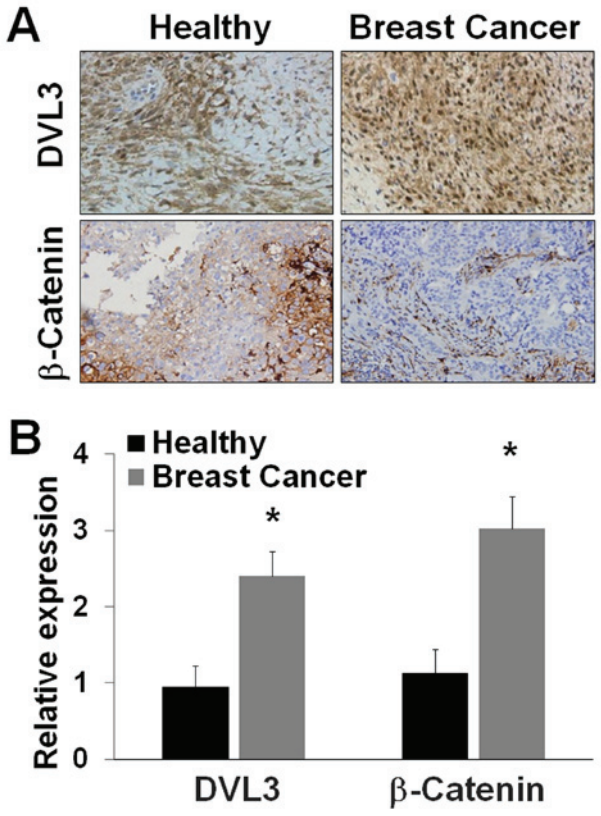

Figure 2. DVL3 and β-catenin are upregulated in breast cancer tissue. (A) IHC analysis of DVL3 and β-catenin in human breast cancer and healthy breast tissue samples. (Magnification, x40). (B) Quantitative analysis of IHC staining of DVL3 and β-catenin in breast cancer and healthy breast tissue samples. IHC, immunohistochemistry; DVL3, dishevelled segment polarity protein 3. *P<0.020 vs. normal.

β-catenin were estimated from tissue sections. DVL3 expression exhibited a >2-fold (P=0.03) increase in breast cancer tissues compared with in healthy breast tissue (Fig. 2B). β-Catenin expression exhibited a >3-fold (P=0.022) increase in breast cancer tissues compared with in healthy breast tissue (Fig. 2B). DVL3 and β-catenin were revealed to be overexpressed in breast cancer tissue compared with healthy breast tissue. The tissue array analysis revealed an increase in the expression of DVL3 and β-catenin in breast tumor samples (Table I). The data obtained were categorized ≤ or >6-fold for a comparative analysis with healthy tissue. A greater level of β-catenin (>6-fold) was noted as statistically significant in breast cancer tissue samples. Statistical analysis revealed that expression levels of DVL3 (P=0.044) and β-catenin (P=0.038) were significantly increased (>6-fold) in breast cancer tissue at an advanced stage of the disease. In addition, the levels of DVL3 and β-catenin were significantly increased (>6-fold) in breast tissue with metastatic cancer (Table I).

DVL3 enhances breast cancer cell proliferation. DVL3 has previously been suggested to act as an important signal transduction molecule, which mediates the Wnt/β-catenin signaling pathway and controls cell growth (3,4). The association of DVL3 and β-catenin signaling in breast carcinogenesis was investigated, in order to examine the role of DVL3 in cell proliferation via Wnt/β-catenin signal pathway activation in breast cancer. MCF-7 and MDA-MB-231 breast cancer cell lines were transfected with a human GFP-tagged DVL3 expression plasmid for stable expression of DVL3. Western blotting revealed that β-catenin was significantly elevated in cells transfected with GFP-DVL3 stable clones (termed GC01 and GC02) (Fig. 3A). GC01 demonstrated a reduced expression of GFP-DVL3, compared with GC02; however, the expression analysis was statistically significant in the two cell lines (Fig. 3B). The XTT cell proliferation assay revealed that MCF-7 and MDA-MB-231 cells with ectopic expression of DVL3 demonstrated a more significant time-dependent rate of proliferation compared with the vector control (Fig. 3B). Furthermore, a clonogenic assay revealed a 1.6-2.5-fold increase in the number of colonies in MCF-7 and MDA-MB-231 cells stably expressing GFP-DVL3 compared with their respective vector controls (Fig. 3C). These results suggested that breast cancer cell proliferation is promoted by DVL3-mediated increases in Wnt/β-catenin signaling activity.

Table I. Clinical and pathological correlations of DVL3 and β-catenin expression in breast cancer tissue.

Characteristics	Total number	DVL3 expression			β-catenin expression		
		≤6-fold	>6-fold	P-value[a]	≤6-fold	>6-fold	P-value[a]
All cases	48	22 (45.8%)	26 (54.2%)		18 (37.5%)	30 (62.5%)	
Cancer stage							
Early	29	12 (41.4%)	17 (58.6%)	0.048	8 (27.6%)	21 (72.4%)	0.041
Late	19	8 (42.1%)	11 (57.9%)	0.044	7 (36.8%)	12 (63.2%)	0.038
Metastasis							
No	35	22 (62.9%)	13 (37.1%)	0.032	19 (54.3%)	16 (45.7%)	0.030
Yes	13	4 (30.8%)	9 (69.2%)	0.022	3 (23.1%)	10 (76.9%)	0.012

[a]Data analysis using χ^2 test; P-values denote comparisons between are compared between ≤6-fold vs. >6-fold; DVL3, dishevelled segment polarity protein 3.

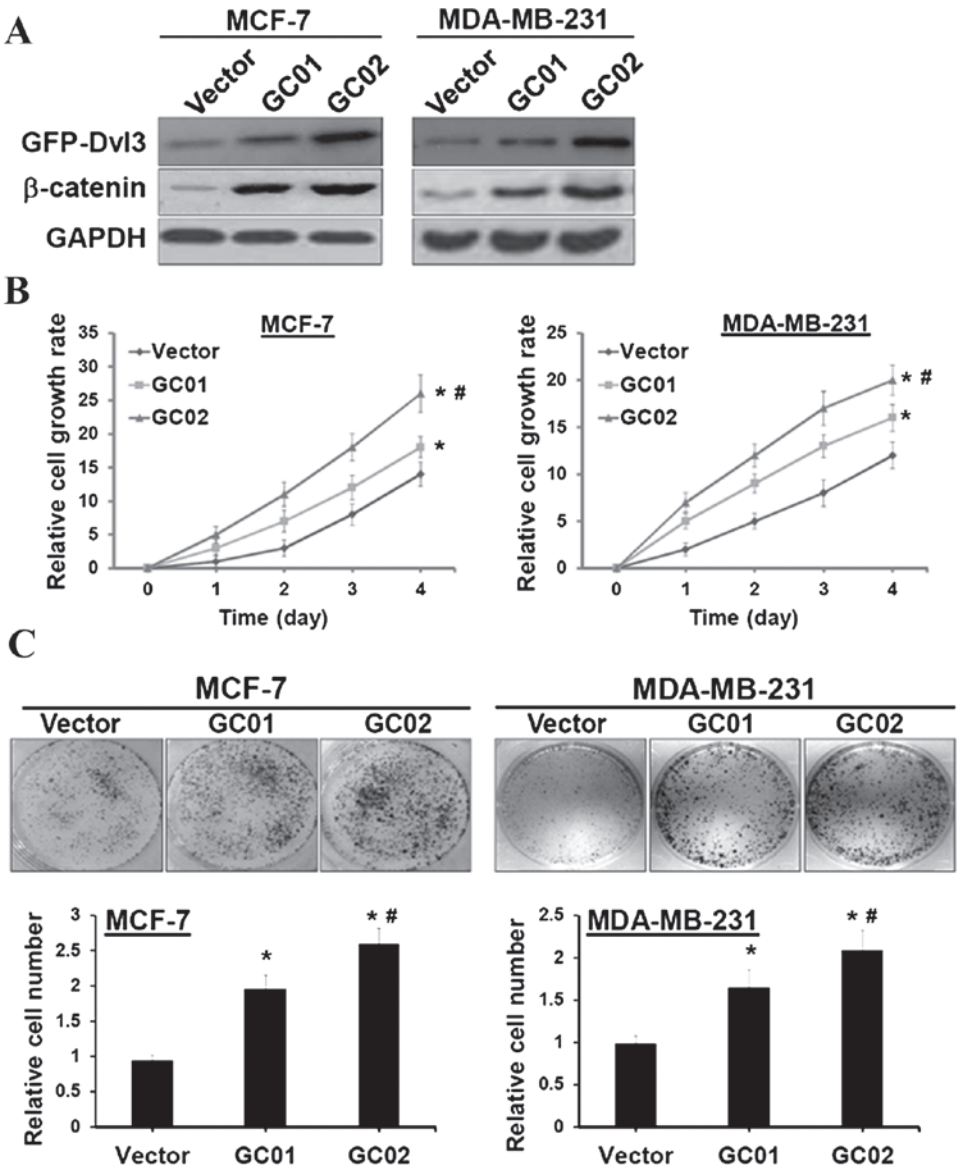

Figure 3. DVL3 ectopic expression enhances breast cancer cell proliferation. (A) Western blot analysis of MCF-7 and MDA-MB-213 cells stably overexpressing GFP-DVL3. GAPDH served as a loading control. (B) XTT proliferation assay of MCF-7 and MDA-MB-213 cells stably overexpressing GFP-DVL3. *P=0.028 vs. vector; #P=0.031 vs. GC01. (C) Clonogenic assay conducted in MCF-7 and MDA-MB-213 cells stably overexpressing GFP-DVL3. *P=0.018 vs. vector; #P=0.021 vs. GC01. DVL3, dishevelled segment polarity protein 3; GFP, green fluorescent protein.

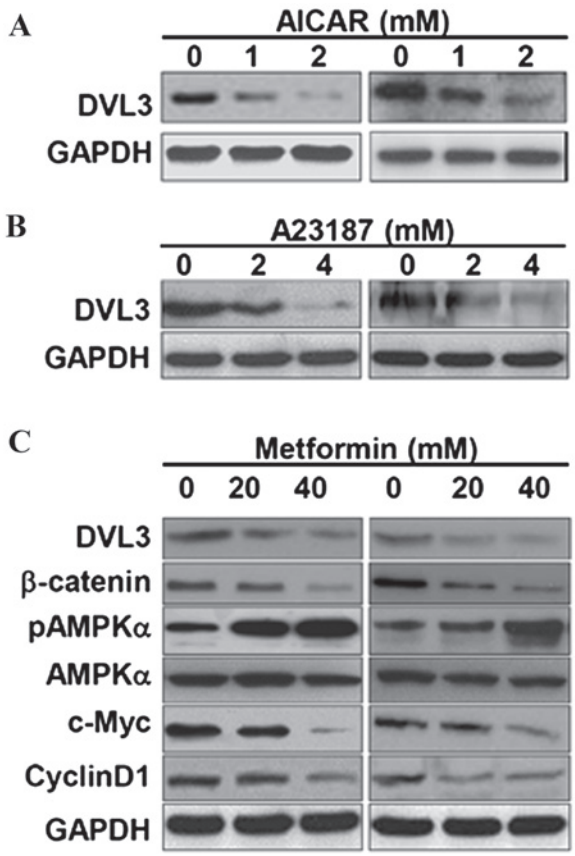

Figure 4. AMPK activators suppress DVL3 expression in MCF-7 breast cancer cells. Western blot analysis of DVL3 expression in MCF-7 breast cancer cell line, following treatment with (A) AICAR and (B) A23187 AMPK activators. Western blot analysis of the expression of DVL3, β-catenin and other proteins following treatment with (C) the AMPK activator metformin. GAPDH served as a loading control. DVL3, dishevelled segment polarity protein 3; AMPK, adenosine monophosphate-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside.

AMPK activators suppress DVL3 in breast cancer cells. It has previously been demonstrated that AMPK activators induce growth inhibitory effects against human cancers, including cervical and breast cancer (13,22,23). The AMPK activators, AICAR and A23187, were used in the present study to analyze the effects of AMPK activation on the expression levels of DVL3 and β-catenin in breast cancer cells. MCF-7 and MDA-MB231 cell lines treated with AICAR demonstrated a marked reduction in DVL3 expression levels (Fig. 4A). A23187, a calcium ionophore, activates AMPK via its upstream calmodulin dependent protein kinase domain by increasing cytosolic calcium levels (24). In the present study, A23187 reduced DVL3 expression in MCF-7 and MDA-MB-231 cells in a dose-dependent manner (Fig. 4B). Treatment of breast cancer cell lines with these two AMPK activators resulted in an inhibition of DVL3 expression levels, which may act to further modulate the signaling events in cancer cell growth. The present study then aimed to decipher the underlying molecular mechanism of breast cancer cell growth inhibition, and its association with DVL3 reduction and AMPK activators by using metformin (another AMPK activator). Metformin is a commonly used AMPK activator and potent anticancer drug (13). Treatment of MCF-7 and MDA-MB-231 breast cancer cells with metformin resulted in a depletion of DVL3 in the cell lines in a dose-dependent manner (Fig. 4C). Treatment with metformin reduced the levels of DVL3 and β-catenin in a dose-dependent manner in the two cell lines. Metformin activated the levels of pAMPKα in a dose-dependent manner, but did not result in alteration to total AMPK levels (Fig. 4C). In addition, metformin dose-dependently inhibited expression of the two downstream transcriptional products of β-catenin, c-Myc and cyclin D1 (Fig. 4C). These results suggested that AMPK activators enhanced the phosphorylation of AMPK, resulting in an inhibition of the DVL3 and β-catenin crosslink, and suppressed cell growth in breast cancer cells as indicated by reduced colony number.

AMPK activator suppresses breast cancer cell growth by reducing DVL3 and β-catenin. The AMPK activators primarily activate AMPK activity; however, it has previously been demonstrated that AMPK activators may be involved in cellular metabolic processes, such as energy metabolism (25,26). The present study subsequently examined whether AMPK activator-mediated DVL3 reduction is dependent on AMPK signaling. Compound C, which is a potent AMPK inhibitor, was used to counteract the effects of metformin on AMPK activation. Treatment of MFC-7 cells with 5 μM compound C resulted in a small increase in DVL3 levels, with a small inhibition of pAMPKα levels (Fig. 5A). Treatment with 20 mM metformin in MCF-7 cells induced a reduction in DVL3 expression and an increase in pAMPKα levels (Fig. 5A). The metformin-induced decrease in DVL3 and increase in pAMPKα levels were reversed following the addition of compound C (Fig. 5A). These results suggested that metformin-induced DVL3 reduction may be dependent on AMPK signaling. It was previously demonstrated that DVL3 ectopic expression may increase breast cancer cell proliferation. The present study elucidated the molecular basis of DVL3 reduction and Wnt/β-catenin signaling by AMPK activators and suppression of cell proliferation. MCF-7 and MDA-MB-231 cells were treated with metformin, and an XTT assay was performed to analyze cell proliferation. Cell proliferation analysis demonstrated that metformin significantly suppressed cell proliferation in MCF-7 and MDA-MB-231 cells in a dose- and time-dependent manner (Fig. 5B). The clonogenic assay supported the results of the cell proliferation assay by demonstrating a reduction in the number of colonies by 41 and 68% in MCF-7 cells, and 22 and 56% in MDA-MB-231 cells at 10 and 20 mM concentrations of metformin, respectively (Fig. 5C). Notably, metformin did not modulate the levels of DVL3 mRNA in the two breast cancer cell lines (Fig. 5D). A very high concentration of metformin (80 mM) resulted in a small decrease in DVL3 mRNA in MCF-7 cells (P=0.042). This observation suggested that metformin may have no interaction with the DVL3 gene and it may act at the post-transcriptional level. These results indicated that breast cancer cell growth inhibition via AMPK is associated with a reduction in DVL3 protein expression levels.

Discussion

The present study demonstrated that DVL3 aberrant overexpression was significantly correlated with increased β-catenin in breast cancer. DVL3 may activate the Wnt/β-catenin signaling pathway and promote growth of breast cancer

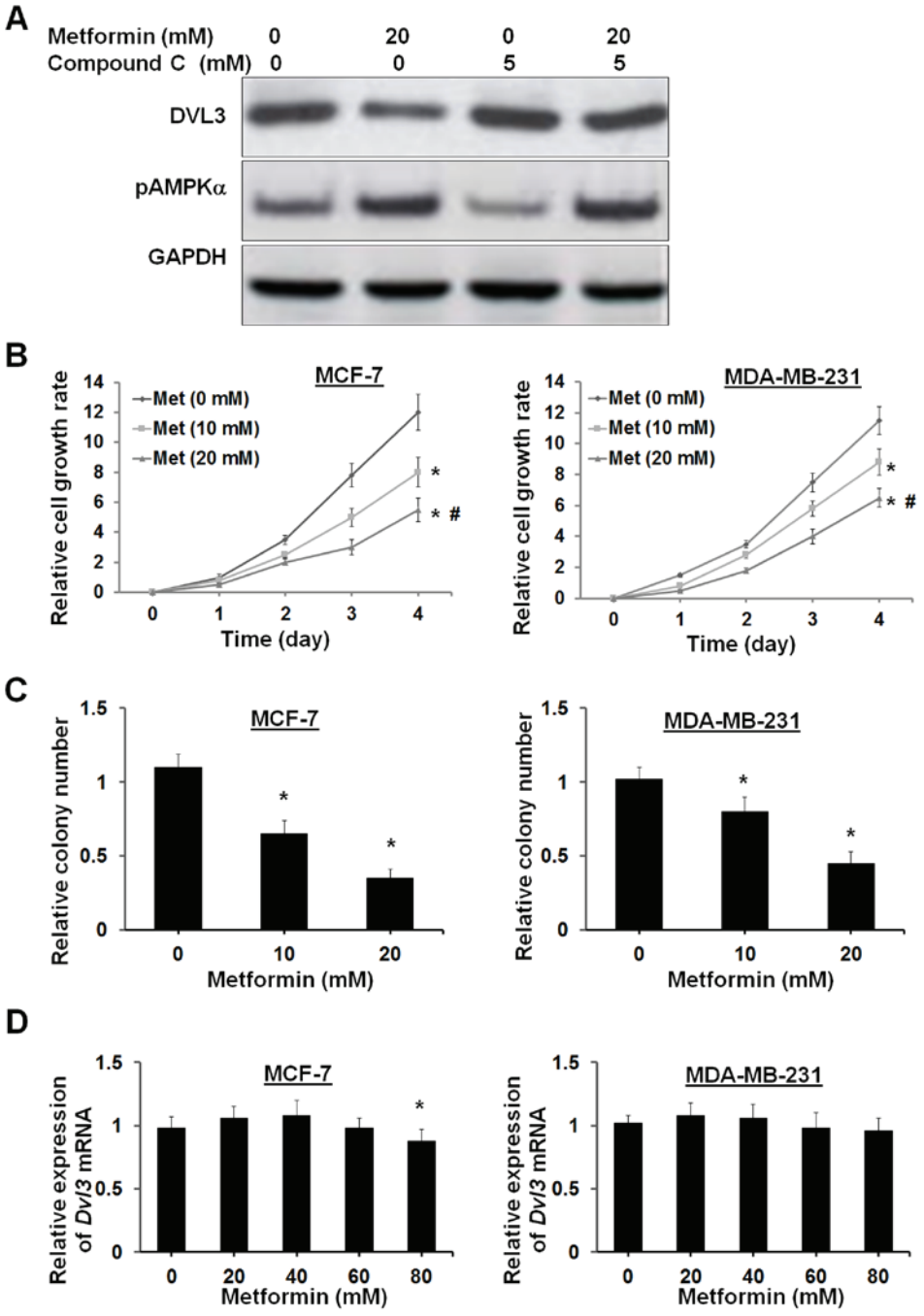

Figure 5. Effects of AMPK activators on DVL3 and breast cancer cell growth. (A) MCF-7 cells were treated with indicated concentrations and combinations of metformin and compound C. Western blotting was performed for analysis of DVL3 and pAMPKα protein expression levels. GAPDH served as a loading control. (B) XTT cell proliferation assay for MCF-7 and MDA-MB-231 cells treated with or without metformin at indicated concentrations. *P=0.022 vs. control; #P=0.028 vs. 10 mM metformin. (C) Clonogenic assay was performed on MCF-7 and MDA-MB-231 cells treated with or without metformin at the indicated concentrations. *P=0.008 vs. control. (D) Reverse transcription-quantitative polymerase chain reaction conducted on MCF-7 and MDA-MB-231 cells treated with or without metformin at indicated concentrations. *P=0.011 vs. control. AMPK, adenosine monophosphate-activated protein kinase; p, phosphorylated; DVL3, dishevelled segment polarity protein 3.

cells. The potential use of AMPK activators in the reduction of DVL3 was investigated to suppress the growth of breast cancer cells. The molecular mechanism underlying the effects of AMPK activators may be an intervention in the interaction between AMPK and the Wnt/β-catenin signaling pathway to impair breast cancer cell growth. The present study primarily demonstrated that AMPK activators exhibit therapeutic potential against breast carcinoma.

The Wnt/β-catenin signaling pathway has been aberrantly activated in various types of human cancer, including breast cancer. It has previously been demonstrated that DVLs, which are positive regulators of the Wnt/β-catenin signaling pathway, are frequently upregulated in several human cancer types, such as lung, prostate, breast, liver and colon cancer (20,27-30). The overexpression of DVLs is associated with an increase in Wnt/β-catenin activity in

human cancer (20,21,31). However, the functional role of the DVL-β-catenin-AMPK interaction in breast cancer remains to be elucidated. The present study demonstrated that DVL3 was significantly upregulated and associated with increased activity of the Wnt/β-catenin signaling pathway in breast cancer cells. However, a similar association was not observed for DVL1 and 2. It has previously been suggested that the upstream kinases of AMPK (including LKB1) are frequently mutated and deleted in various human cancers, including breast cancer (23,32,33). LKB1 reduces AMPK activities, which promote cancer cell growth (8,9). AMPK is therefore a critical target in cancer therapy. It has also been previously suggested that several AMPK activators may suppress cancer cell growth, including breast cancer (13,23,34). However, the molecular mechanisms underlying the effects of DVL3 suppression and AMPK activation on the prevention of breast cancer cell growth remain to be elucidated.

The present study investigated AMPK activation as a potential cancer therapy via the application of the antidiabetic drug metformin (N,N-dimethylbiguanide). Metformin has previously been suggested to inhibit tumor growth via a reduction of serum glucose levels and insulin/insulin-like growth factors, and via intratumoral AMPK activation (34,35). The results of the present study verified the previous findings that metformin administration may reduce cancer formation via AMPK activation, and suggest a possible strategy for the treatment of breast cancer. Metformin was previously observed to reduce β-catenin protein levels by regulating its phosphorylation in human osteoblasts (36). These observations indicated that upstream regulators of the Wnt/β-catenin signaling cascade may be regulated by metformin and thus may control the growth of cancer cells. The present study suggested that AMPK activators may markedly reduce DVL3 expression and subsequently result in breast cancer cell growth inhibition via Wnt/β-catenin signaling. Previous reports using metformin have indicated that its antitumor effects are mediated by the downregulation of cyclin D1 in prostate and breast cancer (37,38). In the present study, the reduction in DVL3 levels was associated with the reduced levels of β-catenin and its downstream targets, cyclin D1 and c-Myc. c-Myc regulates cell proliferation whereas cyclin D1 maintains cell cycle progression; therefore, these targets may be essential transcriptional products of β-catenin in cell growth regulation. Notably, metformin altered DVL3 expression at the protein level, but not at the mRNA level. This result indicated that the regulation of DVL3 mediated by AMPK activators may be dependent on post-transcriptional modification of proteins rather than gene expression regulation. Numerous AMPK activators have been revealed to reduce DVL3, indicating that AMPK activity is essential in regulation of cell proliferation (11,13,22,23,37,38). The effects of AMPK activators on DVL3 expression were assessed by co-treating breast cancer cells with an AMPK inhibitor (compound C) and metformin, to counteract the effects of AMPK activation. Compound C abrogated the effects of metformin on the suppression of DVL3 expression. These results suggested that AMPK activators may suppress cell growth by reducing DVL3 levels, and similar results were observed from *in vitro* cell proliferation assays.

In conclusion, the results of the present study demonstrated that AMPK activators, including metformin, downregulated DVL3 expression and thus reduced the expression levels of β-catenin, c-Myc and cyclin D1, resulting in suppression of cell proliferation. DVL3 is a key oncoprotein, which activates the Wnt/β-catenin signaling pathway and mediates growth of breast cancer cells. A reduction of DVL3 via AMPK activation is important in suppressing tumor development in breast cancer. These results therefore emphasize the therapeutic value of AMPK activators in targeting DVL3 and Wnt/β-catenin signaling in breast cancer treatment. The results of the present study demonstrated that administration of AMPK activators reduced growth of breast cancer cells via AMPK activation, thus suggesting a novel and potential therapeutic strategy for the treatment of breast cancer.

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