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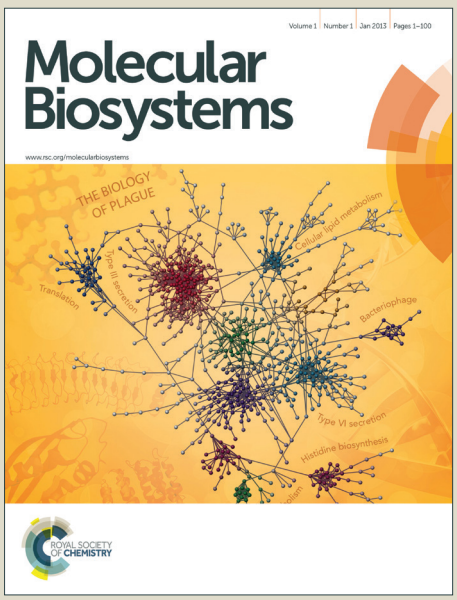

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ARTICLE

Integrated *in silico* and experimental methods revealed that Arctigenin inhibited angiogenesis and HCT116 cell migration and invasion through regulating H1F4A and Wnt/β-catenin pathway

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Key words: Arctigenin, HCT116, Angiogenesis, Cell migration, Cancer thearpy

Arctigenin (ARG) has been previously reported to exert diverse biological activities including anti-proliferation, anti-inflammatory, and antiviral, etc.In the current study, the anti-metastasis and anti-angiogenesis activities of ARG were investigated.To further understanding how ARG played these bioactivities,proteomic approaches were used to profile the proteome changes in response to ARG treatment using 2DE-MS/MS. Using these approaches, a total of 50 differentially expressedproteins were identified and clustered. Bioinformatics analysis suggested that multiple signalling pathwayswere involved.Moreover, ARG induced anti-metastatic and anti-angiogenesis activities were mainly accompanied by a deactivation of Wnt/β-catenin pathway in HCT116 cells.

1. Introduction

Fructus arctii is the dried ripe fruit of *Arctium lappa L.*, also called "Niu bang zi" in China. *Fructus arctii* is a well-known Chinese Tradition Medicine, which mainly contains dibenzylbutyrolactone lignans of arctiin and Arctigenin (ARG, see Fig. 1). Many pharmacological studies revealed that Arctigenin possessed many important bioactivities, including anti-proliferation, anti-virus, anti-inflammation, neuroprotection, inducing apoptosis and ER stress regulation, etc.[1-6] Recently, Li et al. reported that Arctigenin exhibited neuroprotective activity via reducing surplus ROS production and down-regulated the mitochondrial membrane potential.[7] Hsieh et al. revealed Arctigenin changed the expression level of Bcl-2 by p38/ATF-2-mediated histone methylation, resulting the increase of superoxide anion and hydrogen peroxide.[8] Moreover, the Arctigenin induced mitochondrial caspase-independent apoptosis of MDA-MB-231 cells through regulation of the NOX1 and p38-MAPK pathway. Wang et al. combined the Arctigenin, curcumin and epigallocatechin gallate (EGCG) to treat the human prostate cancer LNCaP cells and breast cancer MCF-7 cells, results showed the combination significantly increased the ratio of Bax to Bcl-2 proteins, decreased the NFkB activation, PI3K/Akt and Stat3 pathways and cell migration both *in vitro* and *in vivo*.[9] Most recently, the mechanism studies of Arctigenin towards human hepatocellular carcinoma (HCC) HepG2 and SMMC7721 cells suggested that Arctigenin could induce apoptosis in HCC cells but not in normal hepatic cells, and its apoptotic effect associated with the mitochondria mediated pathway and depend on the decrease of the mitochondrial out membrane potential, up-regulation of Bax, down-regulation of Bcl-2, a release of cytochrome c, caspase-9 and caspase-3 activation and a cleavage of poly (ADP-ribose) polymerase. Moreover, the increased expression of Fas/FasL, TNF-α and the activation of caspase-8 showed that the death receptor related apoptotic pathway was also involved in this process. [10]

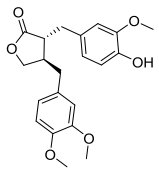

Fig. 1 Chemical structure of Arctigenin.

Although the anti-proliferation and apoptosis inducing mechanisms of Arctigenin were studied well in various cancer cell types.[11-22] However, to our knowledge, inhibition mechanism of Arctigenin on the metastasis, invasion and angiogenesis of tumor cells have not been studied detailed. Most recently, Farge et al. reported that tumorigenic mechanical pressure could potentiallystimulated β-catenin pathways tumor and neighbouring normal tissues, contributing to an unstable positive feedbackloop between oncogene expression and tumor induction,enhancing the growth and metastasis of the tumor.[23]In this study, wearct used HUVEC and HCT116 cell lines as models, combined difference proteomics, *in vitro* and *in vivo* experimental methods to investigate the metastasis and invasion inhibition potential of Arctigenin and the molecular mechanisms involved in this process. Our results demonstrated that Arctigenin could suppressedcellular

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Electronic Supplementary Information (ESI) available: Table S1 as noted in the detailed information of alternated proteins in the proteome analysis. See DOI: 10.1039/x0xx00000x
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migration, invasion *in vitro* and inhibited angiogenesis *in vivo* through down-regulation of Wnt/β-catenin pathway and down-stream proteins. ARG-mediated anti-angiogenesis and anti-metasis effects on the HCT116 cells were triggeredbytheactivationofthe E-cadherin and inhibition of the β-catenin, N-cadherin, MMPs and vimentin.

2. Methods

2.1 Reagents and cell lines

RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM) were obtained from Sigma-Aldrich (St. Louis, MO) and fetal bovine serum (FBS) were purchased from GIBCO (Gaithersburg, MD, USA). Rabbit monoclonal anti-Catenin, anti-Vimentin, anti-MMP-2 and anti-MMP-9 were purchased from Cell Signalling Technology (Beverly, MA, USA). Mouse monoclonal anti-N-Cadherin, anti-E-Cadherin, anti-VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). All the other chemicals and reagents used in this experiment were of highest quality and obtained from standard commercial sources. Arctigenin (ARG) (purity 98%) was purchased from JCKY Institute of Chemical Technology (Beijing, China). A 10 mM stock solution of ARG was prepared in DMSO and stored at -20°C. For all experiments, the desired concentrations of ARG were freshly diluted from the stock with DMEM before use. Human colon cancer cell HCT116 and human umbilical vein endothelial cell HUVEC were obtained from china center for type culture collection. All the cells were incubated in a humidified incubator at 37°C with 5% CO2. Then, cells in the logarithmic growth phase were collected for the following experiments.

2.2 Measurement of cell viability using MTT assay

The cell lines were seeded in 96-well plates at 2500-4000 cells/well (depending on cell type) in DMEM supplemented with 10% FBS and incubated overnight. The cells were treated with different concentrations of ARG or vehicle for indicated durations, and then 20 μL of MTT solution (1 mg/mL, Sigma, USA) was added to each well. After incubation for 2-4 h at 37°C, the formazan crystals were dissolved with 50 μL of acidified SDS (20%, w/v). Absorbance at the wavelength of 570 nm was read in an ELISA reader. Each assay was performed in 3 replicates and all experiments were repeated at least twice. For the HUVEC growth inhibition assay, the cells seeded in 96-well plates (1×104 per well) were starved overnight in EBM2 medium and incubated with Arctigenin for 45 minutes. Then 50 ng/ml vascular endothelial growth factor (VEGF) or 5% FBS was introduced into the assays and the cells were continued to incubate for 72h. The subsequent procedures were performed as described above.

2.3 Cell migration assay

HCT116 cells were cultured to confluence in 24 well plates and wounded using a sterilized yellow pipette tip to make a straight scratch. Cells were rinsed with sterile PBS gently, and then PBS was replaced with DMEM medium containing vehicle or Arctigenin. After incubated 24h, pictures were taken by OLYMPUS digital camera attached to a light microscope.

2.4 Transwell invasion assay

Transwell invasion assay was done as our previously published procedure.[24, 25] In brief, 50 μL per well of diluted Matrigel (BD Biosciences, USA) was added to the Transwell compartments (Millipore, USA), which have been inserted into a 24-well plate. HUVECs were suspended in EBM-2 medium (without growth factors) one hour later and seeded in the upper chamber (3×10^4/100 μL). Afterwards, another 100 μL medium containing vehicle or Arctigenin was added to each upper chamber. The lower compartments were filled with 500 μL EGM-2 medium (EBM-2 medium supplemented with various growth factors). After incubation for 24 hours at 37 °C, the migrated cells were fixed with methanol and stained with 0.05% crystal violet for 15 minutes, followed by rinsing twice with PBS. The cells were photographed under a light microscope (Leica, Germany).

2.5 Angiogenesis in live fluorescent zebrafish assay

Anti-angiogenesis activity of Arctigenin and curcumin was assessed in transgenic zebrash (FLK-1: EGFP) according to the our previously reported procedure.[26-30] Briefly, zebrafish embryos at the 13-somite stage (30 embryos per group) were incubated overnight with vehicle, curcumin or Arctigenin. Then zebrafish were anesthetized and imaged using a fluorescence microscope (Carl Zeiss Microimaging Inc., Germany).

2.6 Two-dimensional poly-acrylamide gel electrophoresis

2-dimensional polyacrylamide gel electrophoresis (2DE) was performed as previously reported with minor modifications.[31-33] Briefly, the total protein extracted as described earlier were dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 0.2% pH3–10 ampholyte, Bio-Rad, USA) containing protease inhibitor (Sigma, P8340). Protein concentration was determined using the DC protein assay kit (Bio-Rad). Each sample containing equal protein contents (1.6 mg) was loaded into an IPG strip (17 cm, pH3–10NL, Bio-Rad) using a passive rehydration method. Separation in the second dimension was performed using 12% SDS-PAGE at 30 mA constant current per gel after isoelectric focusing (IEF) and equilibration. The gels were stained with CBB R-250 (Bio-Rad, 161–0438) and scanned with a Bio-Rad GS-800 scanner. Quantitation of each spot in a gel was determined (OD) using PDQuest software 7.1 (Bio-Rad). Differentially expressed proteins were defined as statistically meaningful ($p < 0.05$) based on both of the following criteria: (1) abundance alterations > 2.0-fold and (2) observed more than three times in the total of four parallel analyses. Four independent runs were made for each paired protein samples to ensure data consistency.

2.7 Network construction

Based on the results of 2-DE analysis, we constructed the protein-protein interaction (PPI) network of the 50 possible target-related proteins of Arctigenin, which was obtained from a database of predicted and experimentally determined PPIs for human, PrePPI.[34] The angiogenesis and metastasis related PPI network of the targets was modified based on Gene Ontology (GO) analysis, which was performed using DAVID database (http://david.abcc.ncifcrf.gov/).[35, 36] And the unified conceptual framework of PPI network was integrated by Cytoscape.[37]

2.8 Function clustering and pathway analysis

Functional enrichment was determined using the DAVID functional annotation tool. GO term related to Biological Process (BP), as well as pathway annotations derived from Kyoto encyclopedia of genes and genomes (KEGG) were used in the functional categories.[38-40] All differentially expressed proteins being in the form of UniProt accession numbers, the functional annotation analysis was performed with the gene ontology tool (GOTERM_CC_ALL). Only those GO terms yielding a $P < 0.05$ using a Fisher's exact test were considered significantly enriched in each gene list while pathways with a corrected $P < 0.05$ were considered significant, classified into hierarchical categories according to KEGG.

2.9 Western blot analysis

For the immunoblot studies, sub-confluent cells were serum starved overnight in EBM2 medium, and then incubated with vehicle, Arctigenin, or curcumin for 24 h. The cells were lysed in RIPA buffer (Beyotime, China) containing Roche protease inhibitor cocktail, after 12,000 g centrifugation at 4 °C for 10 min, the protein concentration was determined by a BCA Protein Assay Kit (CWBIO, Beijing, China). Proteins were separated by gel electrophoresis on 12% SDS-PAGE gels and probed with specific antibodies including anti-VEGF, anti-N-Cadherin, anti-E-Cadherin, anti-Catenin, anti-Vimentin, anti-MMP-2, anti-MMP-9 and anti-β-actin. All of the antibodies were used at a 1:1,000 dilution, and incubated with indicated primary antibodies at 4 °C overnight and horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 2 h, then visualized by using ECL reagents.

3. Results and discussion

3.1 Arctigenin inhibits cell proliferation in HCT116 and HUVEC cells

To investigate the effects of ATG on cancer cells, we examined its cell proliferation effect on HCT-116 and HUVEC cells. As shown in Fig. 2, after exposure of HCT-116 and HUVEC cells to ATG for 48h, the cell proliferation of HCT-116 and HUVEC cells were markedly inhibited in a dose-dependent manner. The IC50 value of ATG was 0.82 μM for HCT-116 cells and 2.51 μM for HUVEC cells, respectively.

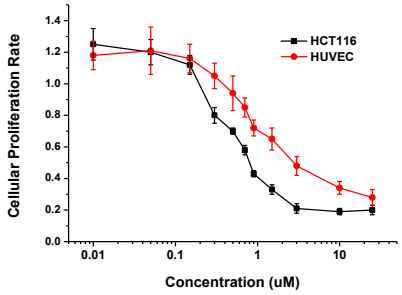

Fig.2 Cell proliferation inhibition effect of ARG on HCT116 and HUVEC cells.

3.2 Arctigenin inhibited HCT116 cell migration *in vitro*

Cell migration is essential for cancer cells in tumor growth, angiogenesis and metastasis[SR2014]. We performed wound healing assays to investigate the effects of ATG on cell migration and observed that 15μM of ATG potently inhibited the migration of HCT116 cells (Fig. 3).

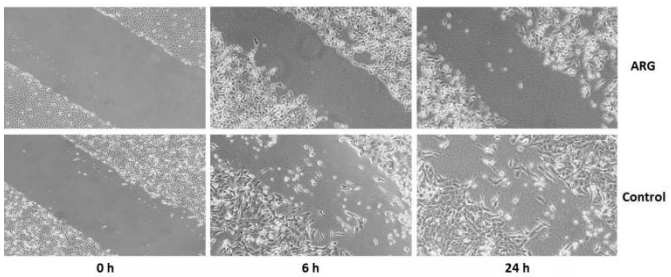

Fig.3 Arctigenin inhibited HCT116 cells migration in wound healing assay. Cells were wounded by the pipette and then treated with 5μM ARG for 24 hours. Migrated cells were quantified by manual counting (50×).

3.3 Arctigenin inhibited HUVEC cell invasion in transwell assays

Cell invasion is essential for cancer cells in angiogenesis and metastasis, in the current study, we performed transwell assays to evaluate the ability of cancer cells to pass through the matrigel and membrane barriers of the transwell in the presence of 15μM ATG or vehicle. Unfortunately, HCT116 cells could not passed the transwell chamber with or without ATG treatment, the HUVEC was chose as substituted models. As shown in Figure 4, ATG obviously suppressed the invasion activities of HUVEC.

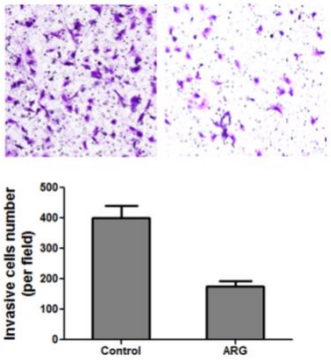

Fig.4ARG inhibted HUVECs invasion in transwell assay. The bottom chambers of thetranswells were filled with 600 μL EGM2 containing various growth factors while the top chambers were seeded with 5×10^4 HUVECs in EBM2 andtreated with different concentrations of compounds for 24 hours. Cells invaded through the membrane were stained and quantified (50×).

3.4 Arctigenin inhibited angiogenesis in transgenic zebrafish assays

To test the anti-angiogenesis effects of ARG*in vivo*, we used a FLK-1 promoter enhanced green fluorescent protein (EGFP) transgenic zebrafish model. We used 30 embryos per group in our study, and each experiment was carried out in three independent replicates. Embryos were maintained in Holtfreter's solution (containing PTU) at 28°C after transplantation procedures, and embryos at fifteen hpf were treated with saline, ARG (5μM or 15μM) or Bestatin (200μM) over night, respectively. As shown in Figure 5, 15μM of ARG considerably inhibited the formation of intersegmental vessels compared with saline-treated embryos, whereas the dorsal aorta and major cranial vessels were not inhibited, indicating the antiangiogenesis effects of ARG*in vivo*.

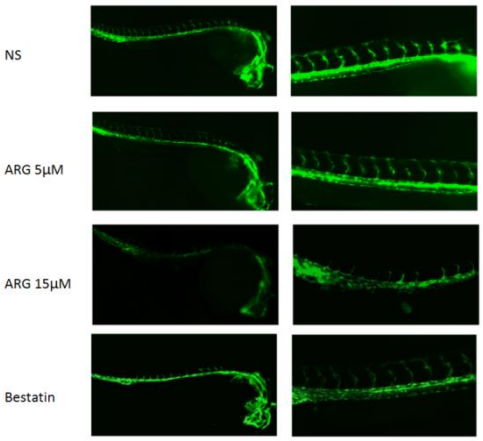

Fig.5ARG inhibited angiogenesis in vivo,fluorescent microscopic images of 30 hpf zebrafish treated with saline, ARG(5μM or 15μM) or Bestatin.

3.5 Differential proteomic profiling of the alterations of proteins

To get an insight into the dynamics of proteins induced by ARG treatment, a representative2DE gel map fromis shown in Fig. 6.Based on PDQuest software analysis, the intensity ratios ofnormalized spots between the gels were calculated. Spotswhich were reproducibly detected in at least threerepeats were subjected to ESI-Q-TOF mass spectrometryanalysis after in-gel digestion. 50 proteins displayed statisticallysignificant (+/− twofold, $p<0.05$) altered expression. Ofthese, 2 proteins were up-regulated and 48 proteins weredown-regulated in HCT116 cells (Table S1).

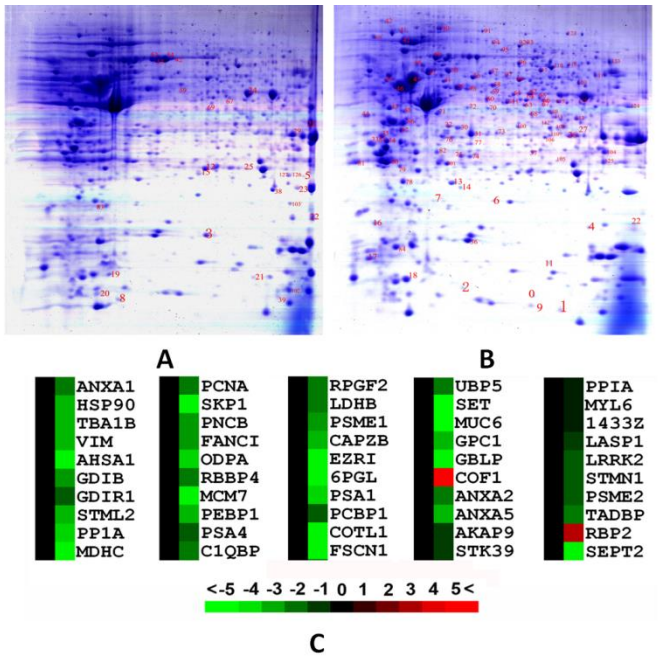

Fig.6 Proteomic analysis of ARG treated HCT116 cells. (A and B) Comparison of proteome profiles bytwo-dimensional gel electrophoresis. Total proteins were extracted from HCT116 cells with(A) or without(B) ARG treatment, then separated on pH3–10 nonlinear IPG strips in the first dimension followed by 12% SDS-PAGE in the second dimension with visualization by CBBstaining. (C) Cluster analysis of proteins expression, in the normalgroup was held constant at 0; up-regulated proteins in ARG treated groupwere shown in red, and the down-regulated proteinsshown in green. The intensity of the green or red color corresponds to the degree of regulation as indicated by the color strip atthe bottom of the figure.

To validate the expression of metastasis and angiogenesis related proteins in ARG treated HCT116 cells, immunoblotting assays were performed using examples of a down-regulated protein (vimentin) and related proteins. We treated the HCT116 cells with 15μM ARG for 24 hours. Whole cell extracts were analysed by immunoblotting for Wnt pathway and angiogenesis markers in the presence or absence of ARG (Fig. 7). The N-cadherin, β-catenin, vimentin, VEGF, MMP-2 and MMP-9 protein expression were down-regulated in HCT116 cells treated with ARG. These results are consistent with a cellular phenotype. In contrast, E-cadherin hadsignificant expression up-regulated with ARG treatment. To our knowledge, HCT116 colon cancer cells have constitutively active Wnt/β-catenin signalling and bearing highly metastatic and angiogenesis potences. Mechanistically, our data now provide evidence that ARG inhibited Wnt/β-catenin signalling leading to anti-metastatic and anti-angiogenesis activities in HCT116 cells directly by components of the Wnt canonical signalling pathway. Our results also illustrated that ARG can significantly suppress protein levels of VEGF and MMPs in HCT116 cells, which were indirectly interacted with Wnt/β-catenin signalling pathway.

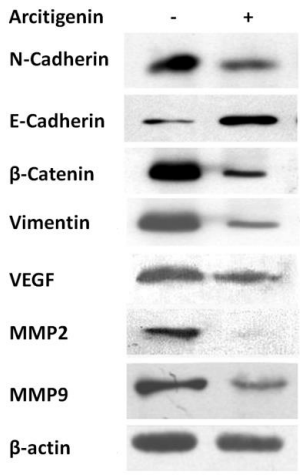

Fig.7 Effect of ARG (15 μM)-induced inhibition of Wnt/β-catenin signalling on the expression of metastatic and angiogenesis markers in synchronized (0 h) and released (24 h) human colon cancer HCT116 cells, as detected by Western blot analysis, β-actin served as a loading control.

3.6 Bioinformatics analysis of identified proteins

Based on the finding of ESI-Q-TOF-MS we have identified 50 proteins which are most significantly regulated by Arctigenin showed in two dimensional gel electrophoresis (2DE). Through PrePPI, the above-mentioned 50 target proteins with 3229 target-related proteins establishing the protein–protein interaction network. The interaction network was composed of 7293 PPIs. In order to refine the above-mentioned PPI-network, we assigned those proteins by functional protein group analysis. Function cluster analysis was used to predict their correlation with angiogenesis or metastasis protein. We assigned those proteins to 1019 annotation cluster, in which 28 functional protein groups related with cell migration, as well as 6 functional protein groups relating with angiogenesis. Consequently, the PPI network was reconstructed into 2 sub-networks. The cell migration sub-network contained 171 proteins forming 378 PPIs, while the angiogenesis sub-network was made up with 91 proteins forming 185 PPIs (Fig. 8A-C).

According to the result, ANXA2 has a directly impact on angiogenesis as well as CFL1, FSCN1, LRRK2, PPIA and RAPGEF2 shares a strong relation with cell migration, based on GO. Meanwhile, a great amount of the target-related proteins showed a strong association with angiogenesis or metastasis, preliminary indicating that as a new anti-angiogenic and anti-metastasis agent, Arctigenin is feasible. Signal network by which Arctigenin induced cell migration and angiogenesis.According to the cell signalling pathway databases (http://sabiosciences.com/pathwaycentral.php or http://www.cellsignal.com/), after importing all the proteins involving in the pathway which relate with angiogenesis or metastasis in the previous PPI network, those subnetworks were further refined to “sub-subnetworks”, which showed that two signalling pathways might be mainly influenced: EIF4E-HIF1A pathway and Wnt pathway (Figure 8D). In EIF4E-HIF1A pathway, EIF4E and H1F1A are inhibited as the downstream protein of the target MCM7, HS90A, KDM5A, ANXA5, EZRI, COF1, GBLP, TADBP are effected. Unsurprisingly, the expression of this pathway, VEGF, also interacts with target proteins LRRK2, HS90A, ANXA2 and TADBP. Meanwhile, mTOR, MAPKK1, RRAS, being proteins of the upstream signalling pathway, PI3K/AKT

pathway and MAPK/Erk signalling pathway, regulated by aiming at target proteins SEPT2, GDIR1, LRRK2, ANXA1, ANXA2, TADBP, MYL6, might inhibit cell angiogenesis.

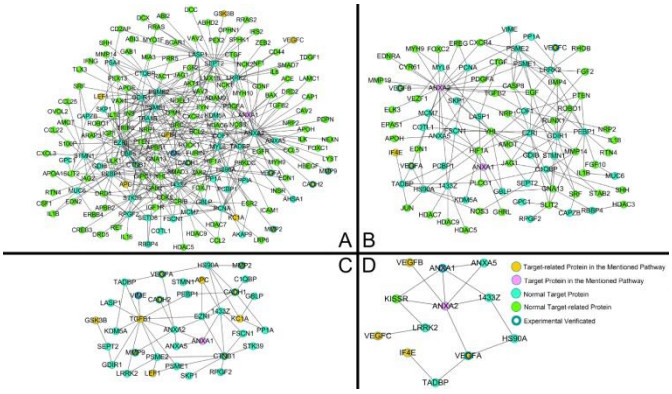

Fig. 8 PPI networks. PPI with proteins involving in the pathway which relate with angiogenesis or metastasis. (A) PPI with proteins relates with cell migration; (B) PPI with proteins relates with angiogenesis; (C) PPI with cell migration related proteins involving in Wnt/β-catenin pathway; (D) PPI with angiogenesis related proteins involving in H1F4A pathway.

Meantime, almost all of the protein (GSK3β, AXIN, APC protein, etc.) in Wnt pathway has been effected by Arctigenin through 23 target proteins, for instance, ANXA1, ANXA2, SKP1, RPGF2, etc，showing its strong force on controlling cell migration. Furthermore, by targeting ANXA2, C1QBP, EZRI, PP1A, PEBP1, HS90A, extracellular matrix proteases (MMP2, MMP9, etc.) may be inhibited with cadherins (E-Cadherin and N-Cadherin) regulated. In our prediction, according to the previous research, the content of the E-Cadherin may increase whereas the content of N-Cadherin may decrease. In addition to that, there are still some other signalling pathways involving in metastasis or angiogenesis, such as SMAD. Nevertheless, due to few proteins in the pathway influenced by Arctigenin, we choose to ignore them.

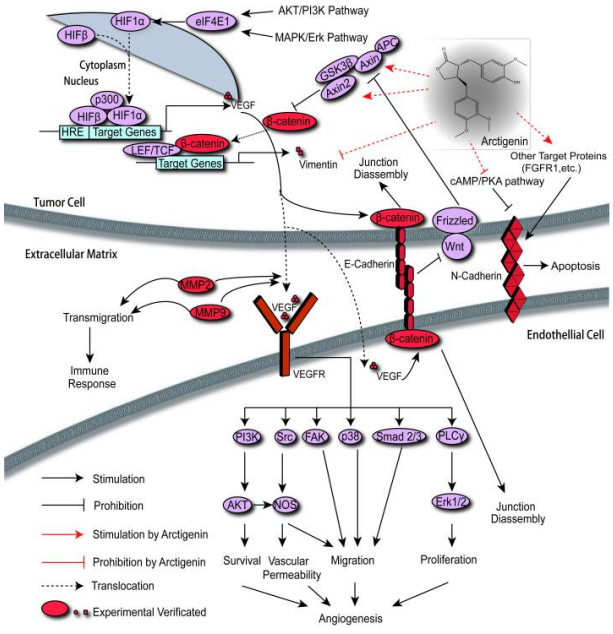

Fig.9 Proposed mechanism of ARG inhibited H1F4A and Wnt/β-catenin signalling pathway leading to the anti-metastasis and anti-angiogenesisactivities.

Conclusions

Arctigenin, as a new anti-angiogenic and anti-metastasis agent, can obviously inhibit angiogenesis, cell migration as well as cell invasion.In summary, we have presented evidence supporting that Wnt/β-catenin signalling pathway is a novel targetof Arctigenin that plays an critical role in the regulation of metastatic and angiogenesisof human colon cancer HCT116 cells (Fig. 9). The proteomic analysis integrated signallingnetwork analysis of the protein data-sets generated revealeddiverse cell biological processes that might directly or indirectlyinteracted with the ARG treatment. In addition to the above-mentioned pathways, Wnt/β-cateninandH1F4A,there aremany otherproteins that are involved in biologicalprocesses including cell angiogenesis and other pathway, such as MAPK. Moreover, a number of extracellular proteins: MMP2, MMP9,E-Cadherin and N-Cadherin were identified in thisstudy. The localization of these intracellular and extracellular proteins maybe performs important functions in the HCT116 cells after ARG treatment. However, the direct binding protein and more precise mechanism of the Arctigenin, and how these drug-target interactions impacton cancer cell proliferation, metastasis and angiogenesis remained unclear thus waited to be elucidatedin future studies.But so far, the performance of Arctigenin in anti-angiogenesis and anti-metastasis have already been pleasantly surprising, thus, we expecting it to play a more important role in anti-cancer therapy in the future.

Acknowledgements

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Notes and references

‡ Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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