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Morin impedes Yap nuclear translocation and fosters apoptosis through suppression of Wnt/β-catenin and NF-κB signaling in Mst1 overexpressed HepG2 cells

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ABSTRACT

Recent clinical and experimental evidences strongly acclaim Yes-associated protein (Yap), a key oncogenic driver in liver carcinogenesis, as a therapeutic target. Of the known multiple schemes to inhibit Yap activity, activation of Mammalian Sterile 20-like Kinase 1 (Mst1), an upstream regulator of Yap, appears to be a promising one. In this study, we hypothesize that morin, a bioflavonoid, mediates its anti-cancer effect through the activation of Mst1/hippo signaling in liver cancer cells. To test this hypothesis, both full length Mst1 (F-Mst1) and kinase active N-terminal Mst1 (N-Mst1)-overexpressed HepG2 cells were used. Exposure of F-Mst1 overexpressed HepG2 cells to morin activated Mst1 by caspase-3 cleavage and thereby inhibited Yap nuclear translocation and fostered apoptosis. Morin suppressed NF-κB p65 and Wnt/β-catenin signaling through Mst1 activation via cleavage and phosphorylation, leading to cell death. Annexin-V/PI staining further confirmed the induction of apoptosis in morin treated F-Mst1 overexpressed cells. The present study shows that morin targets cell survival molecules such as NF-κB p65 and β-catenin through activation of hippo signaling. Therefore, morin could be considered as a potential anti-cancer agent against liver cancer.

1. Introduction

Yes associated protein (Yap), the hippo pathway effector molecule, is pro-proliferative and anti-apoptotic in function. The exacerbated activation of Yap results in increased nuclear translocation of Yap, driving cellular proliferation and survival, ultimately leading to hepatocellular carcinoma (HCC) [1–3]. Hence, Yap was established as an independent prognostic marker for HCC [4–9]. Mammalian Ste20-like kinase (Mst), a core component of the hippo pathway, acts as a negative regulator of Yap and transcriptional co-activator TAZ [3]. Furthermore, several key studies demonstrated Mst1/2 kinases as potent tumour suppressors, and combined deficiency of Mst1/2 kinases led to loss of Yap phosphorylation, massive liver overgrowth and development of HCC [10,11]. Loss of Mst function resulted in Yap nuclear accumulation with accelerated proliferation, resistance to apoptosis and massive organ overgrowth, leading to tumour progression [12,13]. Likewise, activation of Mst affected nuclear and cytoplasmic localization of Yap and TAZ through proteosomal degradation [14,15].

Mst1/2 are closely related, evolutionarily conserved serine/threonine protein kinases playing a pivotal role in cell proliferation, survival, morphology and motility [16]. Mst1, structurally (487AA, 59 kDa) comprised of an active N-terminal kinase region (1-330AA, ~35 kDa). The C-terminal regulatory region is composed of an auto-inhibitory domain and Salvador-Ras association (RA) domain family (RASSF)-Hippo (SARAH) domain [17]. Mst1 contains two caspase-cleavage sites between the kinase and the auto-inhibitory domain [18] and upon cleavage removes two C-terminal nuclear export sequences allowing the truncated N-terminal kinase domain (~35 kDa) to translocate to the nucleus resulting in enhanced nuclear condensation, nucleosomal DNA fragmentation and membrane blebbing [19,20]. Mst1 is activated by various stress and apoptotic stimuli such as UV radiation, staurosporine, hydrogen peroxide and several anticancer drugs [21–23]. Recently, a study showed that Mst1 was predominantly expressed in its full-length form in 76% of HCC tumours, whereas kinase active,

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cleaved Mst1, was basically absent in HCC cells [24]. Interestingly, ~50% of human HCC patients showed aberrant overexpression and nuclear localization of Yap with lower Yap phosphorylation and absence of cleaved Mst1 leading to increased cell proliferation and cell survival [25,26]. All these highlight the importance of activation of Mst1, preferentially for the inhibition of HCC progression.

Morin (3, 5, 7, 2', 4'-pentahydroxyflavone), a bioflavonoid belonging to moraceae family, was isolated as a yellow pigment from almond hulls and old fustic [27,28]. Several reports have acclaimed morin's antioxidant, anti-diabetic, anti-inflammatory, anti-fibrotic, anti-cancer, antihypertensive and neuroprotective activities [29–35]. Also, studies on its metabolites such as morin sulfates/glucuronides showed anti-inflammatory activity against lipopolysaccharide (LPS)-activated macrophages [36]. Previously, from this laboratory, we have shown the anti-hepatocarcinogenic potential of morin in attenuating diethylnitrosamine-induced HCC [37,38]; anti-fibrotic effects in experimental liver fibrosis through inhibition of stellate cell proliferation [39,40]; anti-carcinogenic and anti-inflammatory effects in DMBA-induced mammary carcinogenesis [41–43]. Although morin was reported to inhibit tumour cell growth through regulation of canonical NF-κB and Wnt/β-catenin signaling pathways [37–40,43], its underlying mechanistic action of tumour cell apoptosis is yet to be explored. In this study, we hypothesize that morin mediates its anti-cancer effect through the activation of Mst1/hippo signaling in liver cancer cells.

2. Materials and methods

2.1. Antibodies, reagents and culture media

Morin (purity-95%), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) and Propidium iodide (PI) were purchased from Sigma-Aldrich Chemicals (USA). Dulbecco's Modified Eagle Medium-high glucose (DMEM-GlutaMAX), fetal bovine serum (FBS), Lipofectamine® 3000 transfection reagent and Geneticin® Selective Antibiotics (G418 Sulfate) were obtained from Invitrogen (Grand Island, NY). Rabbit monoclonal primary antibodies such as Mst1, p-Mst1(Thr183), Lats1, p-Lats1 (Thr1079), Yap, p-Yap (Ser127), β-catenin, cyclin D1, NF-κB p65, JNK, p-JNK (Thr183/Tyr185), c-JUN, cleaved caspase-3, cleaved caspase-9, anti-Myc tag and β-actin, mouse monoclonal IκBα antibody and anti-rabbit and anti-mouse HRP conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit monoclonal primary antibodies such as Bax and Gsk-3β, mouse monoclonal Bcl-2 antibody and anti-rabbit FITC green conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Agarose was purchased from GeNei™ (Bangalore, India). All other chemicals and solvents used were of the highest purity grade available.

2.2. Cell culture and morin preparation

Stock cultures of human liver carcinoma (HepG2) cells procured from National Centre for Cell Science (NCCS), Pune, were grown in monolayer at 37 °C in a 5% CO_2 incubator and maintained in DMEM containing 10% FBS. 10 mM solution of morin was prepared in DMSO and diluted in cell culture media as required. The final concentration of DMSO was minimized to 0.1% and this did not affect cell survival.

2.3. Cell viability assays

MTT and LDH leakage assays were carried out to determine the effect of morin on cell viability in HepG2 cells as described earlier [39]. Briefly, $1X10^5$ HepG2 cells/well were seeded in 96-well plates and incubated at 37 °C in 5% CO_2 for 24 h. On 70% confluency, media with different concentrations of morin (1, 2.5, 5, 10, 25, 50, 75, 100, 200, 600 μM) were added and incubated at 37 °C in 5% CO_2 for 24 h and 48 h. The samples included a 'blank' (medium alone) and 'control' (DMSO alone). After incubation, the percentage of growth inhibition (IC_{50}) and LDH leakage (%) were determined.

2.4. Plasmid constructs and transfection

Myc-tagged full length Mst1 constructs (pcMycMst1) in the pcDNA3 (Invitrogen) expression vector were obtained as a gift from Prof. Yukiko Gotoh (The University of Tokyo, Japan). Kinase active N-terminal domain of Mst1 (N-Mst1 (1-330AA)) was constructed by amplification of N-Mst1 from pcMycMst1 by PCR using T7 primer as a forward primer and 5'-CGAATTCTCACATCGTGCCAGAATCCATTTC-3' (1-330AA) as a reverse primer. The amplified fragment (N-Mst1) was digested with *Hind*III and *Eco*RI and subcloned into pcDNA3, as described previously [19].

The plasmids were transiently transfected to $1X10^5$ HepG2 cells/well in a 6-well plate. 1.0 ml of Opti-MEM medium (Gibco™) was added just before transfection and transfection was performed with Lipofectamine 3000 as per manufacturer's protocol. The experiment included Control (untreated HepG2 cells); Full length Mst1 (F-Mst1; transfected with pcMycMst1); F-Mst1+ morin treated (24 h) (transfected with pcMycMst1 and treated with morin (24 h)); kinase positive N-Mst1 (transfected with pcMycNMst1) and vector control (transfected with vector pcDNA3).

2.5. Quantitative Real-time PCR (qPCR) analysis

Total RNA was extracted and each RNA sample was reverse transcribed to corresponding cDNA as described previously [39]. qPCR was performed using SYBR select master mix in StepOne Real-Time PCR system (Applied Biosystems, USA). The primer sequences and amplification condition used are shown in supplementary Table 1. PCR specificity was examined by analysing the melting curves. The data were analysed using the comparative threshold cycle method and represented as fold change in comparison to control HepG2 cells.

2.6. Western blot analysis

40 μg of the protein lysate was separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked for 1 h with 5% non-fat milk powder and incubated overnight with the primary antibodies (anti-Mst1, anti-Lats1, anti-Yap, anti-pMst1, anti-pLats1, anti-pYap, anti-NF-κB p65, anti-IκBα, anti-β catenin, anti-Gsk-3β, anti-Cyclin D1, anti-JNK, anti-p-JNK, anti-c-JUN, anti-Bcl-2, anti-Bax, anti-cleaved caspase 9 and anti-cleaved caspase 3) in 5% BSA solution. Following primary antibody incubation, the membranes were incubated with host specific secondary antibodies for 1 h. Finally, the immunoreactive protein complex was detected by the addition of diaminobenzidine (DAB) or using Enhanced chemiluminescent (ECL) system. β-actin served as loading control. The intensities of the protein bands were quantified using ImageJ software (NIH, Bethesda, USA).

2.7. Enzyme-linked immunosorbent assay of transcription factor NF-κB p65

NF-κB p65 transcription activity in control, morin treated and transfected HepG2 cells were measured in corresponding nuclear extracts (5 μg) using TransAM ® NF-κB p65 protein assay (Active Motif, Carlsbad, CA, USA) as per manufacturer's protocol.

2.8. NF-κB Reporter (GFP) assay

NF-κB Reporter (GFP) assay (Qiagen) was performed as per manufacturer's protocol. In brief, one day before transfection, 2 X 10^4 HepG2 cells per well (96 well plate) were seeded in 100 μl of growth medium with antibiotics to attain 90–95% confluence at the